4-Aza-podophyllotoxins: Synthesis and Use as Anti-cancer Agents

by

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Declaration

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March 2020

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Abstract

Podophyllotoxins, and their derivatives, have shown significant anti-proliferative properties. Etoposide and other semi-synthetic analogues, such as teniposide, have been successfully employed as clinical anti-cancer agents. 4-Aza-podophyllotoxins, which are structurally simplified synthetic analogues of podophyllotoxin, have been successfully synthesised and have shown comparable biological activity to the naturally occurring (-) podophyllotoxin. To further investigate the method of inhibition of this class of compounds, which have been reported to act as either tubulin inhibitors or topoisomerase II poisons, two libraries of 4-aza-podophyllotoxin analogues were synthesised. The biological test results from the first library of analogues was used to inform the synthesis of a second library of compounds. The first library contains a diverse group of twelve 4-aza-podophylltoxin analogues, six with differences in the 4*N*- position. Half of these compounds were modelled after etoposide, and half modelled after the naturally-occurring (-)-podophyllotoxin. This was achieved through *N*-functionalization of the starting material, an aniline, prior to its use in a multicomponent reaction with tetronic acid and a functionalized benzaldehyde. The second library of four compounds was generated using an azide alkyne cycloaddition to modify two alkyne-containing compounds from the first library. The first library has shown IC_{50} values of nano-molar concentrations (125-250 nM) against a WHCO1 oesophageal cell line assay. A small structure activity relationship from these biological results was obtained.

Keywords: Podophyllotoxin, synthesis, anti-cancer, 4-aza-podophyllotoxin, multicomponent reaction.

Opsomming

Podofilotoksien, en hul afgeleides, het beduidende anti-proliferatiewe eienskappe. Etoposiede en ander semi-sintetiese analoë, soos teniposiede, is suksesvol as kliniese teenkankermiddels gebruik. 4-Aza-podofilotoksien, wat struktureel vereenvoudigde sintetiese analoë van podofilotoksien is, is suksesvol gesintetiseer en het vergelykbare biologiese aktiwiteit getoon met (-) podophyllotoxin wat natuurlik voorkom in plante. Twee groepe van 4-aza-podofilotoksien analoë is gesintetiseer sodat die metode van inhibering van hierdie klas verbindings verder ondersoek kan word. Die inligting van die biologiese toetsresultate uit die eerste groep met analoë, is gebruik in die sintese van 'n tweede groep van verbindings. Die eerste groep bevat 'n uiteenlopende groep van twaalf 4-aza-podofilotoksien-analoë waarvan ses verskille toon in die 4*N*-posisie. Die helfte van hierdie verbindings gemodelleer na etoposied en die ander is helfte gemodelleer na (-) podofilotoksien vat in natuur voorkom. Dit is bereik deur *N*-funksionalisering van die uitgangsmateriaal, 'n anilien, voordat dit in 'n multikomponentreaksie met tetronsuur en 'n gefunksionaliseerde bensaldehied gebruik is. Die tweede groep van vyf verbindings is gegenereer met behulp van 'n aziedalkyne-cycloaddition om twee alkienbevattende verbindings uit die eerste groep te verander. Die eerste biblioteek het IC50-waardes van nano-molêre konsentrasies (125-250 nM) teen 'n WHCO1-slukderm-sellyn-toets getoon. Ons het daarin geslaag om 'n klein struktuuraktiwiteitsverhouding te genereer uit hierdie biologiese resultate.

Sletulwoorde: Podofilotoksien, sintese, anti-kanker, 4-aza-podofilotoksien, multikomponentreaksie.

Abbreviations

NMR	Nuclear Magnetic Resonance
MS	Mass spectroscopy
IR	Infrared
GOMOC	Group of Medicinal and Organic Chemistry
TLC	Thin layer chromatography
GC	Gas chromatography
HPLC	High performance liquid chromatography
THF	Tetrahydrofuran
EtOAc	Ethyl acetate
EtOH	Ethanol
DCM	Dichloromethane
MeOH	Methanol
MP	Melting point
FDA	United States Food and Drug Administration
NCI	United States National Cancer Institute
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
RT	Room temperature
MWI	Microwave irradiation
THPTA	Tris(3-hydroxypropyltriazolylmethyl)amine
N_2	Nitrogen gas
CAF	Central Analytical Facility (at Stellenbosch University)
ATR	Attenuated total reflection
UV	Ultraviolet
YSU	Youngstown State University

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Introduction

Podophyllotoxins and their derivatives (compound **1**, see **Figure 1**), are known to have inhibitory activity against various cancer cell lines. Therefore, they have been well researched and form a key part of the field of medicinal chemistry.[1] Previously, podophyllotoxins have been extracted from natural materials, particularly plants from the genus *podophyllum*.[2] The yields for these naturally extracted cyclolignans have been very low, 0.4 – 2.5% mass per mass,[3] which creates the need for fully synthetic analogues. The promising bioactivity exhibited by these compounds resulted in the development of new synthetic strategies to produce the compounds in acceptable yields. Seven podophyllotoxin derivatives have been developed as anti-cancer agents since the structure of naturally occurring (-)-podophyllotoxin was confirmed 1951,[3] and have undergone clinical trials as potential novel clinical treatments. These compounds are: etoposide (**2**[*1]), teniposide, etopophos (currently in use[1]), NK-611, GL331, NPF, and TOP53 (see later in **Figure 5**).[1] There are, however, numerous compounds within this group that remain unstudied, or have shown serious side effects such as severe gastrointestinal symptoms, which made them unsuitable as anti-cancer agents.[*2]

The structure-activity relationship studies of these compounds have shown that they can be heavily modified in certain key areas and remain biologically active, as is the case for etoposide (**2**).

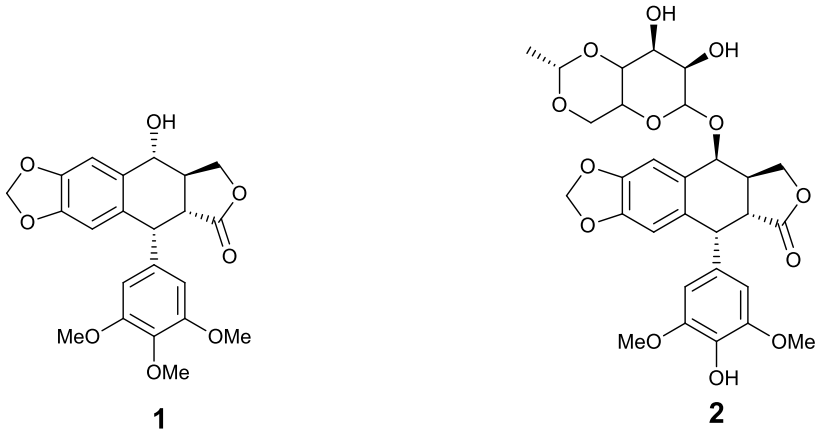


Figure 1: *Structure of (-)-podophyllotoxin, a member of the podophyllotoxin class of compounds (**1**), and etoposide (**2**).*[2,3]

*1 Throughout this text numbers in **bold** are those that have been assigned to compounds that form part of this research.

*2 The introduction and the description of the general instruments and solvents used (in Chapter 3), have been adapted from: Gould, K.T. and van Otterlo, W.A.L Novel synthesis of 4-aza-podophyllotoxin analogues and their potential as anti-cancer agents. *Stellenbosch University Honours paper (Unpublished).* **2017**

(-)-Podophyllotoxin, the naturally extracted cyclolignan, has demonstrated tubulin polymerisation inhibition, while Etoposide (2) and most 4-*N* functionalised 4-aza-podophyllotoxin synthetic derivatives (**Figure 2**) have been shown to be both tubulin inhibitors and/or topoisomerase II poisons. Tubulin, an α-β heterodimer forms the core of microtubules that are vital for mitosis (-)-Podophyllotoxin (**1**) inhibits tubulin polymerisation, which affects the cells ability to divide, and thus inducing apoptosis. [4] Etoposide (**2**) and its analogues, unlike (-)-Podophyllotoxin, have displayed biological activity by acting as a topoisomerase II poison.[5] Topoisomerase II is understood to interact with the supercoiled DNA double helix.[6] By disrupting the action of topoisomerase II, etoposide (**2**) prevents the DNA double helix from re-ligating after uncoiling during the mitotic stage and during protein synthesis. Unable to re-form the double helix, cell death is initiated. Unfortunately (-)-podophyllotoxin (**1**) and etoposide (**2**) induce death in both cancer and healthy human cells. This leads to inefficient treatment of the cancer due to the simultaneous death of normal and cancerous cells.[5]

Synthesis of synthetic derivatives of (-)-podophyllotoxin, has been a stumbling block for many years. This is due to the complex stereochemical elements displayed by the compound. Several synthetic strategies were discussed in a review by Botes *et al.*[7] and Yu, *et al.*,[1] including also the development of one-pot multicomponent reactions (MCRs) used for the synthesis of 4-aza-podophyllotoxins (**Figure 2**).

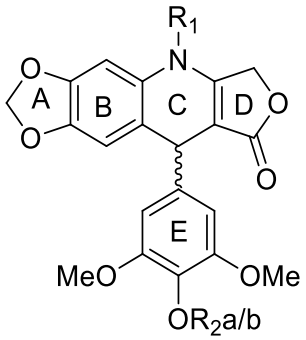


Figure 2: *Generalized structure of the 4-aza-podophyllotoxin analogues synthesized in this project, where R_1 is a series of alkyl groups and R_2 consists of either a phenolic or methoxy moiety*

The research undertaken and described in this thesis was performed with the aim of synthesizing a series of diverse 4*N*-functionalized 4-aza-poodphylltoxin analogues that could act as anti-cancer agents.

These compounds were tested against several cancer cell lines, most significantly an esophageal cancer cell line (WHCO1). The results were used to synthesize a second diverse library. It was hoped that these compounds would yield a new group of potential anti-cancer agents.

This, manuscript consists of five chapters. Chapter 1 provides background information on research into 4-aza-podophyllotoxin analogues. The second chapter presents the results of the syntheses performed for this project. Chapter 3 describes the reactions performed during experimentation. Chapter 4 presents the biological test results obtained for the synthesized libraries. The thesis concludes with a summary of the research and discusses the future lines of inquiry.

Chapter 1

What follows is a discussion on the traits of cancer and current treatment options. At present 60% of all known anti-cancer drugs are derived from natural products.[8] The second section of this chapter briefly discusses the history of natural products, their link to anti-cancer agents and the role natural products play in modern drug discovery. Finally, this chapter discusses podophyllotoxin and the synthesis of 4-aza-podophylltoxin analogues that were the fulcrum of this research.

1.1 Cancer

Cancer is one of the most prevalent killers in the modern world. It is ranked as the 2rd largest cause of death in the human population by the WHO.[9] Almost all of us have had some experience with cancer, either through exposure to a sick loved-one, or ourselves. Approximately 30 years ago the life expectancy of cancer patients after diagnosis was five years. Now the life expectancy of a cancer patient has risen to a 65% chance of survival after five years. Part of the reason for the improved therapeutic outcome is that 47 new and more efficacious anti-cancer drugs were put into production between 2000 and 2010, and 27 between 1990 and 2000.[10]

1.1.1 The hallmarks of malignant cancer

The classic indicators of malignant cancer can be attributed to morphological, molecular and cellular factors, commonly known as the "hallmarks" of cancer.[10] These traits have been extensively reviewed by Hanahan & Weinberg who coined the term in 2000 and reviewed them again in 2017. This section will focus on six characteristics of cancer cells.

i. Self-sufficient growth signaling

Typically, cell growth is carefully controlled, with the number of cells at rest, or in replicative and apoptotic stages remaining in balance. Tumor, or neoplasm formation requires more cells to be in the rest and replicative cell stages than those in the apoptotic stage. This is achieved by self-production of the hormones responsible for cell growth signaling and/or errors in the growth promotion receptors. If either of these occur, a positive feedback loop is created, known as autocrine stimulation. This leads to

continuous growth of cancer cells. One such example is in the SOS-Ras-Raf-MAPK signal transduction pathway.[11,12]

ii. Insensitivity to antigrowth signaling

If the cancer cell shows mutations in the antigrowth hormone receptors it can bypass the checkpoints during the cell cycle that prevent faulty cells from replicating. These checkpoints occur between the G1 and S, S and G2 and G2 and M phases of the cell cycle. Typically, a healthy cell will assess whether to continue towards mitosis at these checkpoints. In cancer this does not occur, and mitosis is almost always instigated as a result of bypassing these checkpoints.[10]

iii. Apoptosis evasion

During normal cell growth, if a cell does not receive pro-survival signals from the extracellular matrix or incurs damage to its DNA, apoptosis will be triggered. The cell will then begin to die, beginning with the disruption of the cellular membranes and ending with the cell being engulfed by the phagosomes, tissues and cells around it. This process will usually be completed within 24 hours in normal human cells. Cancer cells can evade this through various mutations of either the proapoptotic (Bc1-2 family), anti-apoptotic receptor proteins, or by inactivation/deletion of the p53 tumor suppressor genes.[10]

iv. Unlimited replicative potential

Healthy human cells, unlike cancer cells, have limited proliferative potential due to the shortening of the telomeres on chromosomes. Once a healthy cell achieves this limit of approximately 60-70 divisions it will undergo senescence due to the inability to completely replicate the 3’ end of the DNA strand during the S phase of the cell cycle. Cancer cells can evade this through expression of hTERT, a telomerase catalytic subunit, avoiding the shortening of the telomeres.[11]

v. Sustained angiogenesis

Tumors, like normal tissues require the flow of nutrients, and this is facilitated through blood vessels. Human tissues typically only induce the growth of blood vessels during wound healing and commonly only through endothelial tissues. To facilitate blood vessel growth, tumors secrete growth factors

inducing healthy endothelial, and other related tissues, to facilitate angiogenesis. The healthy cells will therefore produce blood vessels that will feed the tumor.[13]

vi. Tissue invasion and metastasis

This is the most dangerous part of cancer, causing 90% of deaths due to cancer. Cancer cells can spread to unrelated and surrounding tissues by moving through the extracellular matrix or blood vessels. These pioneer cells can then establish secondary tumors in other parts of the body or tissues surrounding the original tumor.[11]

1.1.2 Chemotherapy

There are typically three methods of treatment for cancer, namely: radiation therapy, chemotherapy and surgery. While surgery and radiation therapy are crucial methods, the most important is chemotherapy. Tumors can be removed with surgery, however, frequently reduction of the size of the neoplasm is required before surgery, which is achieved through either radiation therapy or chemotherapy.[14]

Surgery cannot treat all cancers, for example leukemia, and can be risky to the patient depending on the location of the tumor.[15] Surgery can also not treat the dispersion of small cancer cells caused by metastasis, thus requiring it to be used with radiation and/or chemotherapy. Like surgery, radiation, and ionizing radiation, has met with success in the treatment of localized cancers. Though radiation can be accurate, it will almost always damage the healthy cells around the tumor.[15] Radiation treatment, however accurate, has similar if not worse side effects, such as sterility, nausea, hair loss, and diarrhea, when compared to chemotherapy. Because of these effects, chemotherapy is often the preferred method of treatment. It not only helps to manage malignant tumors, but can also target cancer cells throughout the body and can be adjusted based on the type of cancer, as well as its susceptibility to different chemotherapeutic agents.[14] While the other methods are still in use and are viable treatments, this section will focus on chemotherapy for the treatments of cancer cells.

Ideally chemotherapeutics should be selective for cancer cells and leave the host undamaged, however, due to the currently insufficient understanding of the chemistry and biology of the human body this is almost impossible. In 2018, the Nobel prize for chemistry was awarded for antibody targeting for

autoimmune diseases.[16] It is hoped that the methods described by Frances Arnold, George Smith and Gregory Winter will open a new future for anti-cancer research. However, until this can be achieved on large-scale and human genome sequencing costs decrease, the need for conventional small molecule anti-cancer agents will remain.

There are typically two main small molecule chemotherapeutic drug development tactics, the first being cytotoxins, the second targeted therapeutics. Cytotoxins often lead to broad cell death, and frequently target multiple sites. For the most part these drugs have been developed and put into practice without their mechanisms of action being thoroughly understood. Targeted therapeutics instead aim to only impact one specific intracellular target site, such as tyrosine kinase inhibitors. They have been developed using molecular in silico screening, high-throughput screening or protein-based approaches.[10] The cytotoxic drugs currently in use are often discovered from natural products using the P338 mouse model *in vivo*, or cellular screening methods. Cytotoxins have been found to mostly target microtubules, damage DNA or inhibit DNA synthesis.[10]

Some cytotoxins which exhibit mechanisms that are not thoroughly understood, and exhibit serious side effects like hair loss or gastrointestinal toxicity, are still used to treat cancer as they are effective.[14] Targeted therapies are also effective; however, drug resistance is often acquired, and mutated cancer cells within a tumor that are not sensitive to the desired target site will continue to proliferate. Due to these conflicting benefits and drawbacks, drugs from both cases are often employed together. Treating cancer in a patient can often involve the simultaneous use of five to six different chemotherapeutics to treat one patient.[10] Due to the diversity of the human genome and their respective cancers, until our technological, chemical and biological understanding improves, we will require a diversity of anti-cancer agents.

Podophyllotoxin and its analogues are an example of a cytotoxin; however, as research into the field of podophyllotoxins has improved, their mechanism of action has been uncovered, and we can begin to blend targeted therapeutics with conventional cytotoxins as a way forward. Since more than 60% of currently discovered anti-cancer agents are inspired by natural products, like podophyllotoxin,[17] it is imperative that we continue to explore this resource for more potential anti-cancer agents.

1.2 Natural products

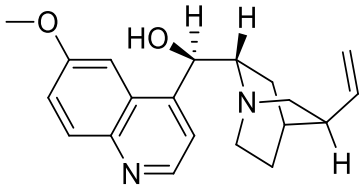
Humans have a long history of using natural products in medicines. Some suggest the use of natural products as medicine extends beyond our recorded human history, going back almost 60 000 years. In 1975 a paper published by Solecki suggested that pollen deposits at a Neanderthal grave site indicated their early understanding of the medicinal properties of plant materials.[18] This section discusses compounds that can be included under the natural products banner; the history of natural products in medicines; and their importance to modern medicine. It concludes by discussing podophyllotoxin, the inspiration for this thesis, and how podophyllotoxin came to be a prevalent part of modern anti-cancer technology.

1.2.1 History of natural products

Natural products can be broadly described as components of living organic matter that humans can use. This encompasses everything from paper and cotton, to penicillin. This chapter will exclusively focus on those natural products that have medicinal value. It has been theorized that many natural products that are used in medicine stem from secondary metabolites. This mainly includes compounds that are not necessary for the primary growth and development of the organism,[12] but rather compounds that either allow an organism to thrive, outcompete other species, or make use of excess nutrients.[19] Despite this understanding, the role of secondary metabolites is not clear in many organisms, unlike microbial production of anti-biotics. There is still much debate as to why organisms would develop these components, and why so many of them are medicinally useful. The most popular theories center around co-evolution, recently the theory of xenohormesis.[19]

In 1975, scientist Ralph S. Solecki discovered pollen deposits at a Neanderthal burial site located in Northern Iraq. The bodies recovered at the site showed the presence of at least seven species of plant, including plants of the genus Achillea (Yarrow), which is known for its medicinal properties. Solecki suggests that presence of these plants, and others with medicinal properties, could point towards early medicine. While it is uncertain that this was the intention of early humanity, there is no doubt that humans have a long history of using natural products in medicine.[18]

For many years the biology of disease was not well understood, even now our understanding has many gaps. Initially our understanding was patchy and rudimentary at best. Many early drugs such as opium, alcohol, salicylic acid (**5**) and quinine (**4**) predate modern medicine (see **Figure 3**).[20] One of the oldest records of human history dates to ancient Mesopotamia (2600 BC). It depicts in cuneiform, on clay tablets, the use of natural products to cure a variety of ailments and diseases. Other historical records, such as *The Chinese Materia Medica* from 1100 BC, and Erbers Papyrus from Egypt 1500 BC, further depict humanities' use of natural products. These records refer to thousands of plants and their potential medicinal uses. Other such records come from ancient India (Ayurvedic hymns, 1000 BC), ancient Greece (*De Materia Medica,* 100 AD*)* and other parts of the world.[21,19] Many encyclopedias and books have since been written, such as the works by the Persian philosopher Avicenna, the *Cannon Medicinae*, summarizing Greek and Roman medicine of the time. The *Pen-ts' as kang mu in* (1596 AD) by Li Shih-Chen records 1898 herbal drugs and 8160 prescriptions from China.[21]



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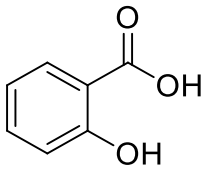

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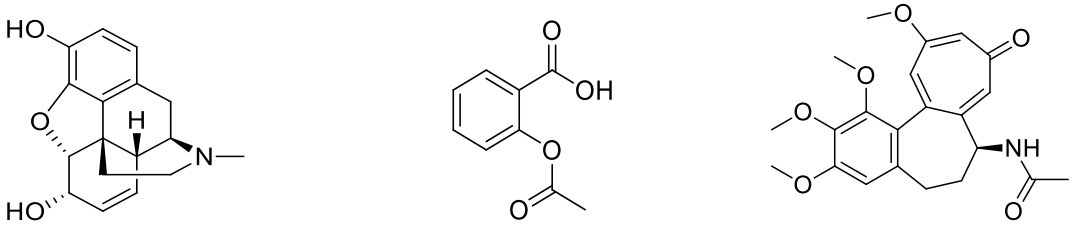
Figure 3*: The structure of quinine (**4**) and salicylic acid (**5**).*

In the 16th century German medical doctor and natural scientist, Paracelsus, otherwise known Phillip van Hohenheim, formulated the "Doctrine of Signatures" in which he suggested that the physical appearance of drugs can be used to identify drugs, and states that each disease has a corresponding cure to be found in nature. Though these ideas are no longer considered valid, they are the beginnings of rational drug discovery.[20]

In the early 1800s American colonists' lack of easily accessible medicinal knowledge sparked the era of household medicine. Various almanacs and publications such as Samuel Thompson's *Thompson's New Guide to Health* were made available to the general public and gained popularity.[21] Humans have used

these products as either wraps for wounds or as chewed herbs, consumed products or even in smoked form. It has only been in the last century that we have made use of synthetics in commercial medicines, starting with the use of morphine (**6,** see **Figure 4**), derived from opium in 1805 and made commercially available in 1826 by Merck.[22] The first semisynthetic to be marketed was aspirin (**7**) produced by Bayer in 1899.[21]



6 **7** **8**

Figure 4: *The structures of morphine (**6**), aspirin (**7**) and colchicine (**8**).*

Natural products currently play a significant role in medicines, specifically in chemistry and the -omics fields. Structure elucidation has played a very large role in this transition, specifically allowing chemists to synthesize compounds based on natural products. This transition to synthetic medicines brought with it a decrease in the cost of medicine, thus inspiring the use of synthetic compounds in medicine, e.g. the discovery of colchicine (**8**).[4]

The first step away from fully natural medicines was characterized by the complete isolation of compounds, e.g. quinine (**4**). Quinine was originally sold and prescribed in its impure form, as bark form Cinchona trees and shrubs. In the 1800's Pelletier and others purified and isolated quinine allowing for its use as a medicine in the treatment of malaria. Quinine is still in use as an anti-malarial medicine, and malaria has shown surprisingly little development of resistance to the drug.[12]

Natural products and anti-cancer chemistry became coupled in the 1950's when, under Dr Jonathan Hartwell, the US National Cancer Institute (NCI) recognized their potential. Many compounds have since been discovered and utilized with NCI funding and support.[23]

Modern medicine, specifically in drug discovery, still derives many ideas from natural sources, but our direct use of natural products has decreased greatly, the largest influencing factor being obtainable yields of pure compounds from their natural sources. The use of modern technologies such as combinational

chemistry and biosynthetic pathway manipulation, have also lead to a decrease in the prevalence of the use of natural products both as extracts and semi-synthetic products.[23]

Semi-synthesis involves the modification of compounds starting with the natural product to increase its medicinal efficacy. Typically this involves compounds obtained from microbial sources as they often produce large amounts of starting material.[12] The key drawbacks of semi-synthetics are the necessity for the natural product starting material; the need for chemical selectivity during synthesis; and, finally the purification of the product, resulting in low yields.[12] Etoposide is a good example of successful semi-synthesis.[24]

As time progressed, there was a move away from direct extraction to fully synthetic alternatives. This was observable across multiple fields, including the perfumery industry. It was not until the popularized use of Chanel No. 5 in 1921, that synthetic compounds became widespread in industry. Natural products still do provide of a large portion of potential drug leads.[25]

1.2.2 Natural products as medicines in modern drug discovery

It is assumed that at least 60% of current medicines are derived from natural products,[26] and we have only explored less than 10% of our total biodiversity for biologically active compounds useful to modern medicine, suggesting a massive untapped resource.[22] The same can be said of anti-cancer agents, with more than 60% of current anti-cancer agents having been inspired by natural products.[27]

Just over 100 years ago due to the ideas of Fischer, and the work of Langly & Ehrlich, work towards rational drug design was further improved through the suggestion of the “lock and key” principle. This suggested the idea that certain cells have specific receptors that can act as hosts for drugs, and that one need only modify the drug until it suits the target receptor This limited the creativity of drug design, as emphasis was placed on designing drugs for a particular receptor. Since then this has been further elaborated and adjusted with increased knowledge of protein and enzyme structure and their behaviors in space, as well as how they fold to accommodate ligands.[20]

Omics technologies are currently favored for target site discovery and the synthesis of more targeted drugs. These include genomics, proteomics, shell interfering RNAs and mouse gene knockout models.[28]

Currently target discovery methods can be summarized as either ‘molecular’ or ‘systems’. While the molecular methods encompass *in vitro* studies and observations of the mechanics within the cell, or the disease, molecular methods focus on identifying very specific intracellular targets and are used extensively in oncology. Systems methods are focused on the disease, within the entire host organism, and include *in vivo* studies. They are widely used in epidemiology and pathology.[20]

Despite these methods of drug target identification, the need for drug diversity is still high and this is where natural products shine. Though most natural products discovered today will not go on to be used immediately to generate a lead compound, they do offer a starting point, which, using structure activity relationships we can tweak to suit our needs. SAR and Qualitative SAR (QSAR) are the medicinal chemists’ best tools for finding new potential drug leads. When harnessing these tools several things need to be kept in mind specifically ADME-Tox, bioisoterism, stereochemistry and the Lipinski rule of five. These aspects are further outlined by Krogsgaard-Larsen *et al.*[20]

1.2.3 Current themes in Natural Products Chemistry

The invention and application of HPLC-MS, GC-MS, two-dimensional chromatography, and other modern chromatographic separation techniques has made the discovery of natural product-based drug leads significantly easier. This coupled with crystallography, used for protein identification, and NMR spectroscopy can make targeted drug discovery even easier as the database begins to grow. Each further step towards the improvement of separation and identification techniques makes the discovery of new drugs faster and more efficient and when coupled with high throughput-screening makes drug discovery even more achievable.[28,29,30]

We are, however, still dependent on biological assays, as only 2% of the overall bacteria in the world can be cultured in a laboratory, even with modern screening techniques. This creates a gap in the potential for discovering new drug leads to combat harmful microbes.[32] This said, microbes are also gaining

popularity as a source of potential drug leads and are even currently in use in biosynthesis.[1] Understanding the human microbiome has also shown potential for practical use in treating cancer. A review by Whisner & Athena Aktipis discusses research aimed at determining the link between cancer cells and the human microbiome. Their research states that microbes and cancer cells can co-evolve within the body and may even share nutrients with each other.[33]

Alternatively, molecular modeling can be used to predict potential drug leads. Once the target site has been identified the tools discussed can be used to narrow down potential modifications of the molecule. This method is also currently in use alongside genomics and proteomics to make new potential individualized drugs.[34]

Natural product chemistry still offers the potential for undiscovered cures or treatments. However, as we slowly improve our understanding of the chemistry behind disease we can begin to use modeling to predict how to cure disease, without the need for broad spectrum screening, and move towards targeted synthesis, and the use of various -omics techniques.[35]

1.3 Podophyllotoxin and its analogues as anti-cancer agents

Podophyllotoxin and its analogues have been extensively researched and reviewed over the last century, particularly since the identification of its structure in 1951 by Hartwell and Schrecker.[3] Research into podophyllotoxin and its analogues have been extensively reviewed by Yu *et al*. between 2003-2007, 2008-2010, and most recently in 2017.[1] There have also been extensive reviews into 4-aza-podophyllotoxin analogues, their uses and total synthesis one such review was done by Botes *et al*. in 2014 on the synthesis of 4-aza-podophyllotoxin analogues by MCR.[7] This section gives a brief overview of the research performed since podophyllotoxins' isolation from Podophyllin and focusses on the work towards the fully synthetic derivatives, with a targeted focus on 4-aza-podophyllotoxins.

It has been reported that plants containing podophyllotoxin, specifically those from the *Podophyllum, Juniperis* and *Diphylleia* genus were used as medicines by the indigenous populations of North America and the Himalayas in the form of an aqueous extract.[1,3] The first written reports of these plants being used as medicines as dates as far back as 1731.[3] Podophyllin, in the alcoholic extract from

rhizome of *Podophyllum* was first reported in the United States Pharmacopeia in 1820, and it was then referenced again in the English Pharmacopeia in 1864.[3,36] The extract, podophyllin, became commercially available in the 1850s as a medicine, with it being prescribed, among other things, as a purgative, cathartic and anthelminthic. There are also reports of it being used as a rudimentary anti-cancer agent in India in the 1700s.[3]

Between 1942-1946 naturally-occurring (-)-podophyllotoxins' mode of action became more thoroughly understood, and it was removed from the United States Pharmacopeia due to its toxicity.[36] It was not until its structure was identified chemically in 1951, and proved by a total synthesis, done by Gensler *et al.*,[37] that it began to be researched as a potential drug lead. This has since lead to a plethora of studies into its mode of action, structural modification, semi-synthesis, total synthesis and even commercial application.[1]

Since 1951 seven semi-synthetic analogues have made it to late stage clinical trials: etoposide (**9**), teniposide(**10**), etopophos (currently in use[1]), NK-611 (**12**), GL331 (**14**), NPF (**13**), and TOP53 (**15**) (currently in clinical trials).[1] Etoposide (aka Vepesid®, **9**), its prodrug etopophos (**11**), and teniposide (**10**) are commercially used and approved by the FDA as anticancer agents (see **Figure 5**).[6,36] Podophyllotoxin itself has also been commercially marketed under the names "Condoline" and "Warticon" as creams to treat genital warts.[38] Furthermore, podophyllotoxin and its analogues have shown significant anti-cancer activity, however, this is not the full extent of its uses. Podophyllotoxin and its analogues have been approved as antivirals (mainly in treatment of the Human papilloma virus (HPV)), in dermatology (in the treatment of venereal warts), as vascular disrupting agents, and as an arthritis medication.[27] Podophyllotoxin analogues have been extensively used to treat Wilms tumors, non-Hodgkin and other lymphomas, colon and lung cancer.[17] Further research has also shown the best responses occurred when podophyllotoxins were used in combination with cisplatin.[27]

(-)-Podophyllotoxin (**1**) shares a target site with colchicine, with both drugs inhibiting tubulin polymerization[39] which affects the cell's ability to divide, and thus induces apoptosis. Tubulin, an α-β

heterodimer, forms the core of microtubules, and is vital for mitosis. We now know that the affected cells enter mitosis, but cannot undergo chromosomal separation, leading to cell death.[27,4]

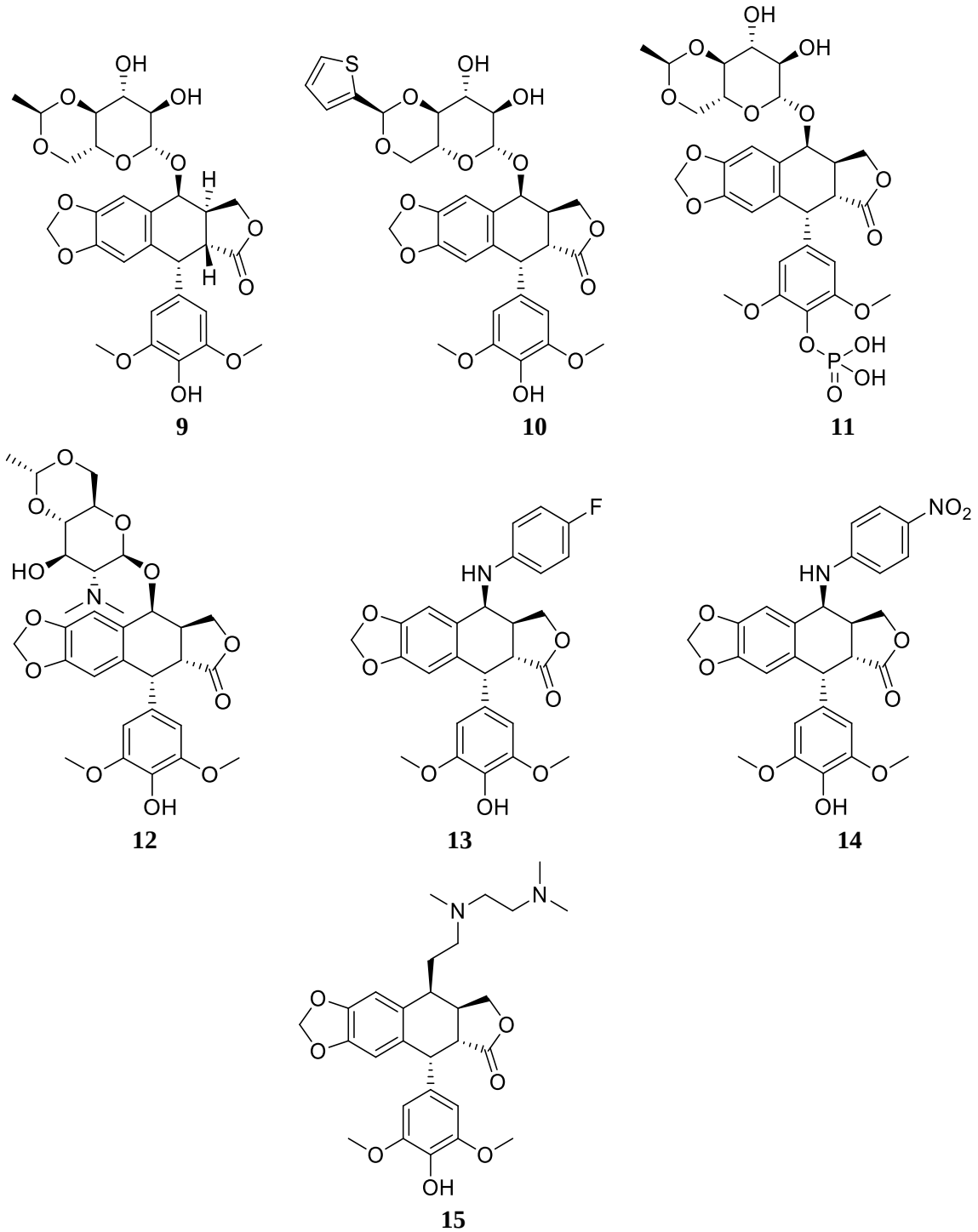
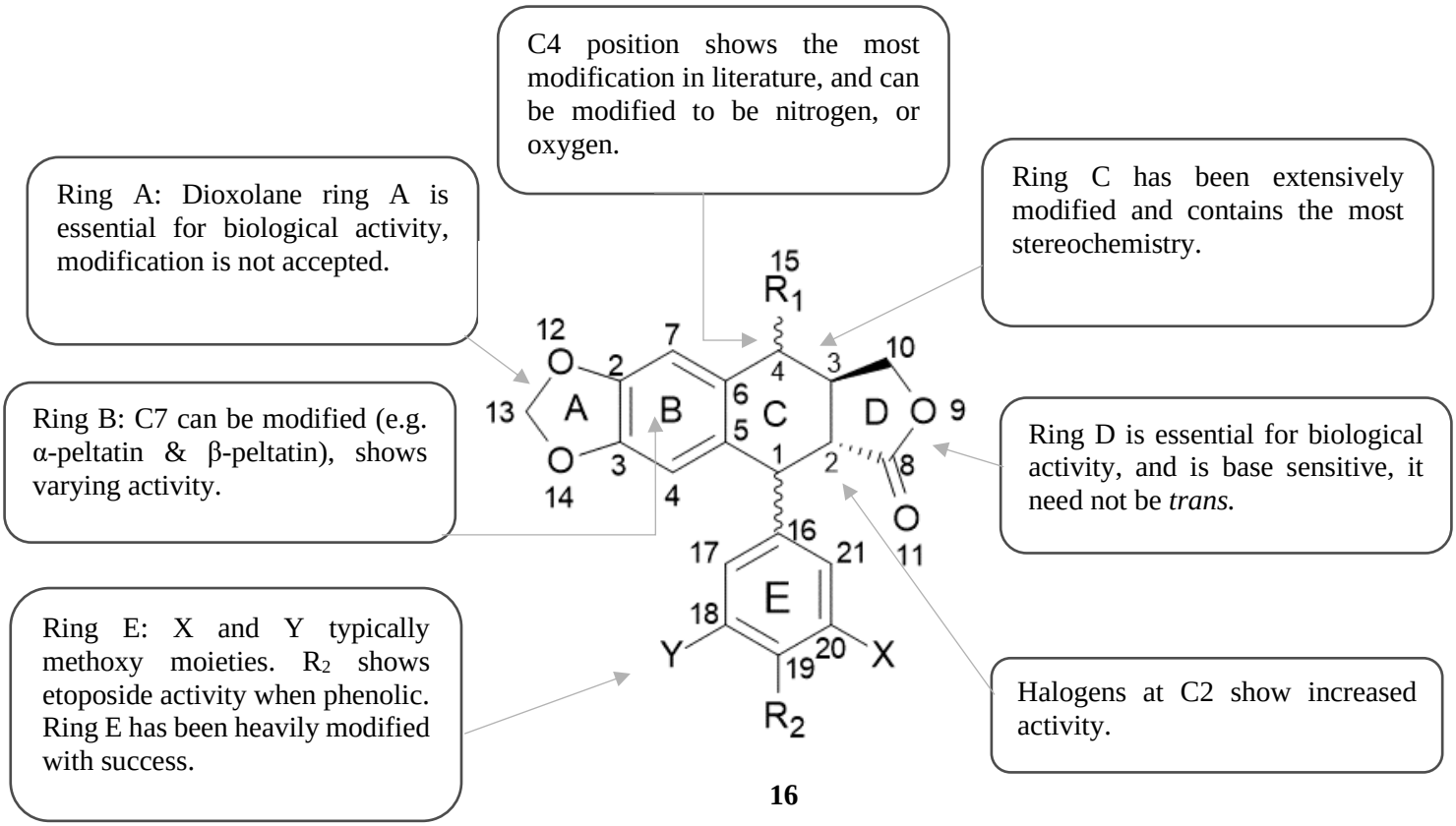


***Figure 5**: The structures of Etoposide (**9**), Teniposide (**10**), Etopophos (**11**), NK-611 (**12**), NFP (**13**), GL331 (**14**) and TOP53 (**15**)*

Etoposide (**9**) has displayed bioactivity by acting as an irreversible topoisomerase II inhibitor. Cancer cells will in fact not even enter mitosis one hour after administration of the drug.[27,5] Topoisomerase II is understood to interact with the supercoiled DNA double helix.[6] By disrupting the action of topoisomerase, etoposide (**9**) prevents the DNA double helix from re-ligating after uncoiling during the mitotic stage, and interferes with the protein synthetic processes. Unable to re-form the DNA double helix, cell death is initiated. Podophyllotoxin (**1**) and etoposide (**9**) induce death of both cancer cells and healthy cells.[39] They act as anti-cancer agents based on the principle that cancer cell metabolism is faster than that of healthy cells. This leads to inefficient treatment of the cancer due to the simultaneous death of normal and cancerous cells.[5] Other antineoplastic mechanisms have also been reported for GL331(**14**), etoposide (**9**) and other analogues. These mechanisms have been attributed to the formation of radical species after the drug enters the cell. There have also been reports of interactions with tyrosine phosphate and several kinases.[36]

Podophyllotoxin is classified as an aryltetralinlactone cyclolignan and is comprised of a flat ridged five ring system. This system contains four main points of stereoisomerism (C1-4 on ring C). There are many iterations of podophyllotoxin (see **Figure 6**) and its extracted analogues, including deoxpodophyllotoxin (hydrogen at C4 instead of alcohol), 4'-demethylpodophyllotoxin (phenolic group in *para* position on E ring, C19), α-peltatin (alcohol functional group on B ring instead of C ring, C7) , β-peltatin (alcohol functional group on B ring instead of C ring, C7, and phenolic moiety on C19) podophyllotoxone (aldehyde at C4).[1]



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Ring A: Dioxolane ring A is essential for activity. Opening of the ring, or functionalization of the phenolic groups (12/13) has not shown improved cytotoxic activity.[1] Research has also been done into replacement of Rings A and B to accommodate a naphthalene system.[40]

Ring B: Though this ring does accept modification, modification has not shown improved activity against Topo II when compared to etoposide (e.g. α-peltatin & β-peltatin).[36]

Ring C: This is the most modified ring. Nitrogen at C4 constitutes a 4-aza-podophyllotoxin. Note that in **Figure 5** this position experiences the most variety, this is also seen in the naturally extracted compounds. The C1 position gives rise to diastereomers, these diastereomers show indistinguishable biological activity.[1]

Ring D: The *trans* lactone ring D has been extensively modified with varying success, *cis* lactone, lactam, hydroxy acids and various heterocycles and open ring modification is occasionally accepted. Ring opening to hydroxy acid can occur *in vivo* leading to inactivation.[1,36]

Ring E: These have experienced extensive modification; it should be noted that this ring need not be able to rotate.

Figure 6: *Generalized structure of podophyllotoxin and summary of the attempted modifications to the structure and corresponding biological activity changes.*

As can be seen in **Figure 6**, the most common point of modification is on the C4 position of the C ring. The C4 position has been modified in total synthesis to incorporate nitrogen or oxygen atoms in place of carbon at this position. It is also this position and the para position on the pendant E ring that have seen successful modification with corresponding activity changes (**Figure 5**). It has been postulated that the para position on the E ring may be responsible for mechanistic changes in biological activity.[36] Before 2008 there were four main methods used for synthesis of (-) podophyllotoxin, all of which showed low yields that lead to the continued use of the naturally extracted product for the synthesis of many analogues. The lack of synthetic options originates from the base sensitive *tans* lactone ring D. The synthesis of GL331 was the first two-step one-pot reaction to afford produce drug from the natural product.[41]

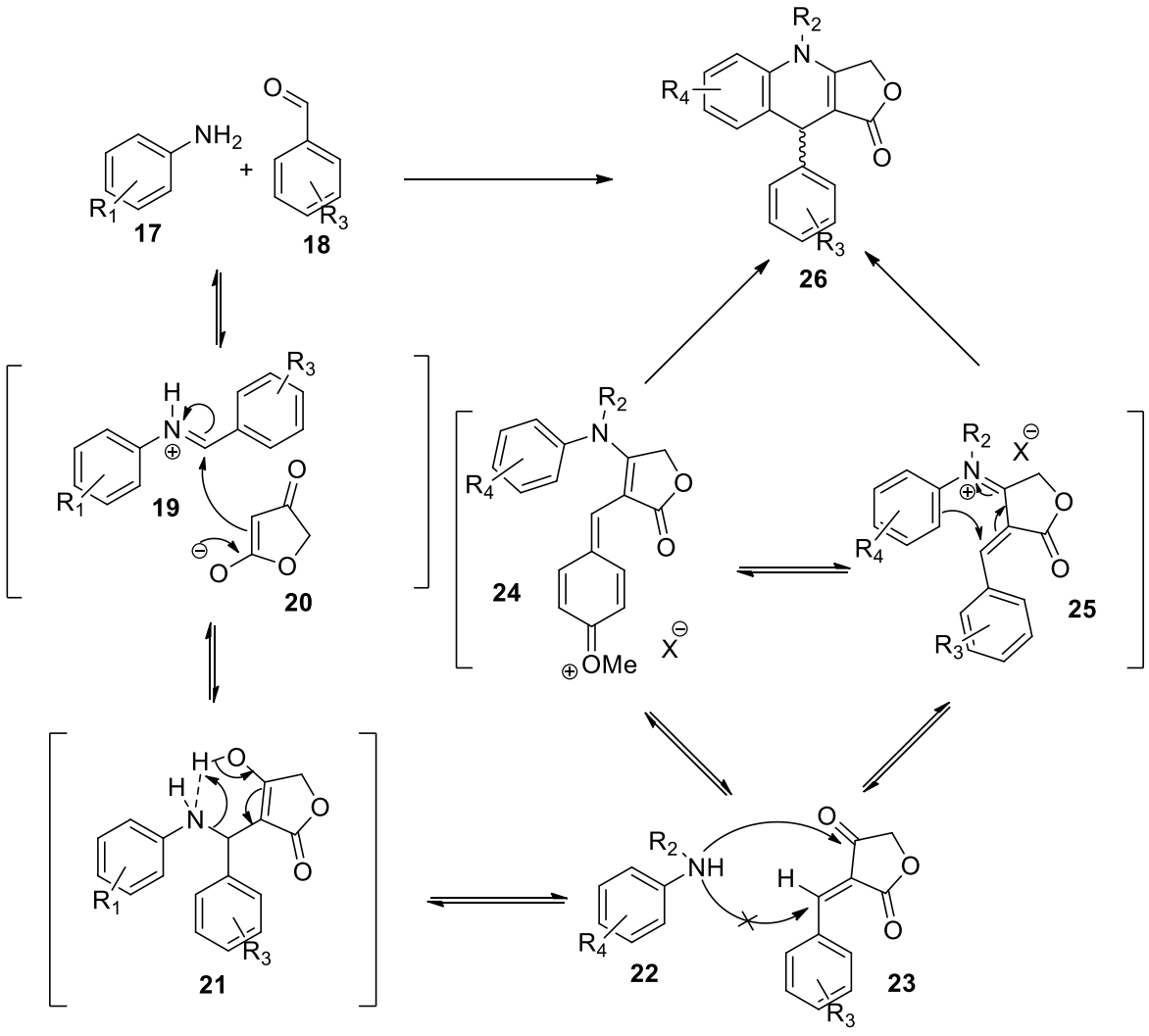
Despite the difficult of synthesising (-)-podophyllotoxin extensive research into podophyllotoxin and its analogues has been performed, therefore focus has been placed on the research into 4-aza-podophyllotoxin analogues. Though both 4-aza and 4-oxy show significant loss of stereochemistry at the C4 position, several studies into the activity of these modified compounds have shown comparative acidity to natural podophyllotoxin and etoposide. It should be noted that the biological activity of the 4-aza-podophylloptoxin was undertaken with racemic mixtures of the analogues. Natural podophyllotoxin and etoposide show IC_{50} values of between 0.076 and 0.05 µm against HeLa cell lines, which is similarly reported for the 4-aza-podophyllotoxin analogues.[1] Detailed information on the various IC_{50} values have been reviewed by Yu et al.(2017).[1] It should be noted that a significant research step was taken towards the total synthesis of (-)-podophyllotoxin and was undertaken in 2013 by Ishikawa and co-workers and consists of 10 steps.[7] The most recent total synthesis of (-)-podophyllotoxin in six steps was performed by Renata and co-workers in 2019.[42]

1.3.1 4-Aza-podophylltoxin analogues

The first reported total synthesis of 4-aza-podophyllotoxin analogues cannot be accurately traced, however, in 2000 Takeya and coworkers reported a three-step synthesis of two 4-aza-podophyllotoxin analogues, which was considered the most significant step towards short total synthesis. In 2002 a one-step,

one-pot-MCR was developed by Giorgi-Renault and coworkers.[37] This reaction was done in ethanol using a functionalized aniline, tetronic acid and a functionalized benzaldehyde to provide the desired structure.

Since the development of the MCR procedure in 2002, investigations into fast and simple synthesis of 4-aza-podophyllotoxin analogues have been undertaken. The mechanism for this reaction has been a topic of much debate, with the currently most accepted mechanism proposed by Jeedimalla *et al.*[43] This depicted below in **Scheme 1**.



***Scheme 1**: Scheme to show the mechanism proposed by Jeedimalla et al. in 2013.*[43]

The synthesis of 4-aza-podophylltoxin analogues can be divided into two parts, the first the formation of functionalized anilines, and the second the multi-component reaction. The 4-aza-

podophylltoxin structure is formed through the formation of a Knoevenagel adduct (**19**), followed by the addition of tetronic acid (**20**), regenerating the aniline (**17**). The functionalised aniline (**22**) then undergoes 1,2 addition to form a condensation product (**24**/**25**) that can then undergo ring formation to produce the 4-aza-podophylltoxin (**26**). Jeedimalla *et al*. also propose the use of a sacrificial aniline, included here as **17**, like *p*-chloro-aniline to increase yields of the desired analogue **26.**[43] There has recently been another extensive study of the mechanism which proposes that the exact equivalents of the starting materials used can have a major effect on the structure obtained.[44]

There still exist a wide range of podophyllotoxin analogues that have yet to be explored. It is hoped that this project will help to take one step forward into the future of treating cancer and using our understanding of the natural world to do so.

Chapter 2: Results and Discussion

2.1 Aims and Objectives

This research had two key interests. The first was to synthesize the modified 4-aza-podophyllotoxin-analogues (see **Figure 7**) through a set of multicomponent reactions to further investigate structure activity relationships. The second was to add biologically active moieties to the 4-*N* position that can selectively target cancer cells, or act as fluorescent markers. By achieving these two objectives cell apoptosis would hopefully be induced through selectively poisoning topoisomerase II and/or through tubulin inhibition by the 4-aza-podophylotoxin as discussed in **Chapter 1**.

These aims were accomplished through the generation of a library of *N*-substituted anilines. The anilines were used in multicomponent reactions with functionalized benzaldehydes and tetronic acid following the method proposed by Giorgi-Renault and co-workers in 2002.[37] The functional groups were added with the intention of overcoming the problems experienced with previously tested 4-aza-podophyllotoxins-analogues, i.e. severe gastrointestinal side effects and mild insolubility.[45] The products synthesized were sent for biological activity testing, the results of which were to be used to motivate the synthesis of more selective podophyllotoxin-analogues.

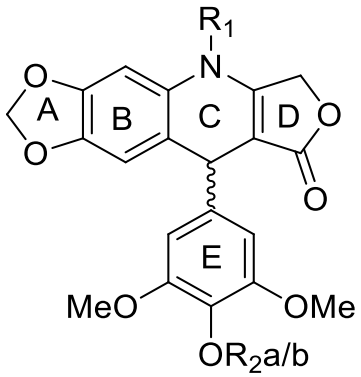


Figure 7: *Generalized structure of the 4-aza-podophyllotoxin analogues synthesized in this project, where R_1 is a series of alkyl groups and R_2 consists of either a phenolic or methoxy moiety.*

The research was undertaken in two parts resulting in: Library 1 and Library 2. Library 1 was synthesized with the goal of testing various modifications on the C4 position on the C ring (**R_1**, **Figure 7**), as well as modifications to the lower E ring, specifically to **R_2** (**Figure 7**). Library 2 was synthesized with the intention of further modifying the **R_1** position of compounds synthesized in Library 1. This was achieved by using click chemistry on compounds **36a** and **36b**. Twelve final compounds were synthesized and isolated in Library 1 and a further four in Library 2. Biological data obtained from testing compounds in Library 1 (see **Chapter 4**) was used to inform the synthesis of Library 2.

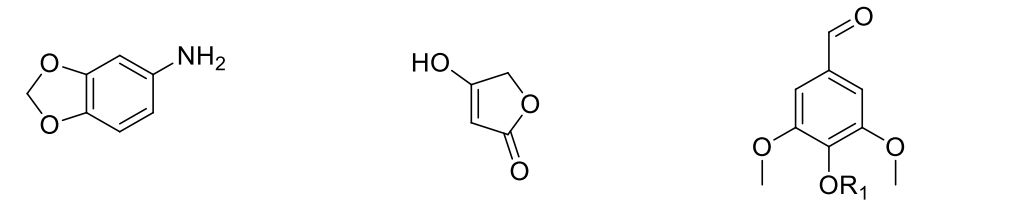
This chapter will first provide details of the syntheses undertaken. The synthesis of Library 1 is described, starting with the synthesis of *N*-functionalized anilines, followed by the synthesis of 4*N*-functionlized-aza-podophyllotoxins, and finally the synthesis of modified compounds from Library 1. Library 2 is then discussed. Library 2 was synthesized from products found in Library 1 and a set of azides. The section discussing Library 2 begins with the synthesis of the azides and ends with the 4-aza-podophyllotoxin click reaction products.

2.2 Synthesis of Library 1: modification of the *4N*-position through the synthesis of N-functionalized anilines

The choice of which 4-aza-podophyllotoxin analogues to synthesise for Library 1 was motivated by four factors. First, the 4-aza-podophyllotoxins that were synthesised needed to have potential for further modification on the 4-*N* position. Second, the analogues needed to have the ability to mimic podophyllotoxin analogues that act as topoisomerase II poisons, currently in chemical trials or in use, specifically etoposide and GL-331, through the lower E ring substitution and *4N*-substitution. Third, the *N* functionalized anilines should not interfere with the MCR. Finally, the 4-aza-podophylltoxin analogues generated needed to be sufficiently dissimilar in the 4-*N* and R_2 positions to generate a structure activity relationship.

2.2.1 Synthesis of anilines for MCR in Library 1

The main starting material chosen for this project was *3*,4-(methylenedioxy)aniline as it forms the A, B and part of the C rings (see **Figure 7**). The following anilines were synthesized in yields sufficient for synthesis of the desired 4-aza-podophyllotoxin analogues:



SM1
3,4-(methylenedioxy) aniline

SM2
tetronic acid

SM3
syringaldehyde (**a**) or 1,2,3-timethoxybenzaldahyde (**b**)

Figure 8: *Starting materials (SM) used in the synthesis of the 4-aza-podophylltoxin analogues.*

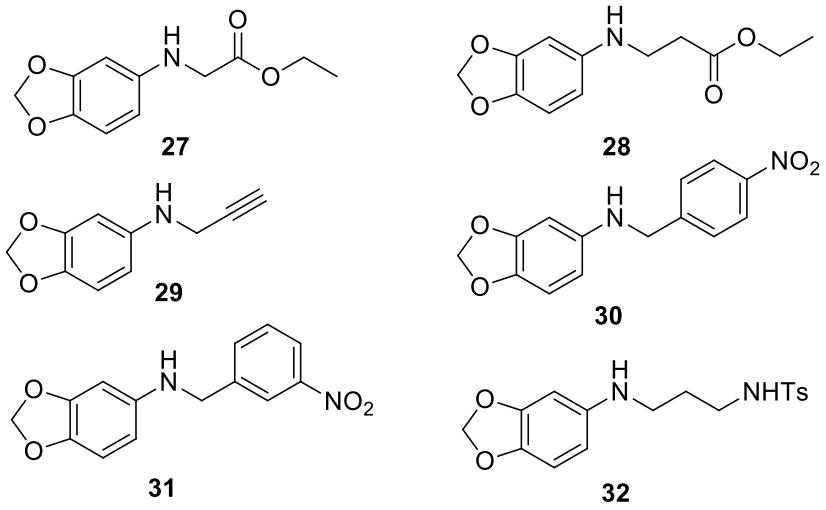
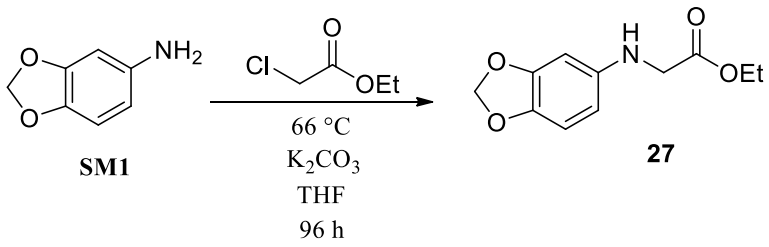


Figure 9: *Functionalized anilines synthesized from 3,4-(methylenedioxy)aniline.*

Ethyl benzo*[d]*[1,3]dioxol-5-ylglycinate (**27**) was successfully synthesized, by heating for 96 hours at 66 °C in THF, using ethyl chloroacetate and K_2CO_3, affording the product in a 51% yield. K_2CO_3 was chosen instead of a stronger base, as its use decreased the potential for bis-alkylation. Ethanol was initially used as solvent but afforded a much lower yield (17%). This was most likely due to hydrogen bonding interference, between the ethanol and the amine nucleophile during the reaction (bis-functionalization was observed in

the minor product). Options other than the classical S_N2 strategy, such as protection and then deprotection of the primary amine, or use of reductive amination, would be inefficient. Protection and deprotection could only yield the product over three steps, instead of one as displayed here (see **Scheme 2**). Reductive amination would have resulted in multiple side-products. Protecting groups would have led to longer, inefficient reaction schemes.

The ^{1}H NMR spectrum for compound **27** showed the appearance of a new triplet peak at 1.28 ppm, a singlet peak at 3.83 ppm and quaternary peak at 4.23 ppm, not present in the spectra of the starting material (**SM1**), indicating that compound **27** was synthesized. In addition, MS data showed a dominant peak at 224.09 *m/z* as expected.



***Scheme 2**: synthesis of methyl ester functionalized aniline*

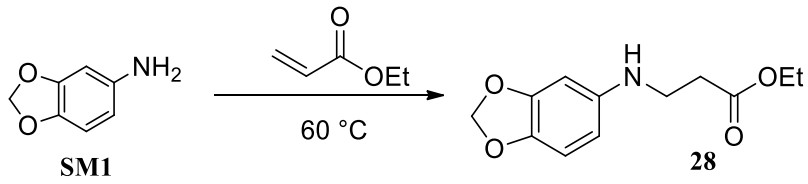
Table 1 shows how the optimization of the synthesis of compound **27** was achieved. As can be seen in the table, reactions 1and 3, in ethanol, afforded significantly lower yields than those performed in THF. It was clear that increased reaction times led to increased yield for the first 72 hours (48%, reaction 4, **Table 1**). After that increasing reaction times did not lead to a significant increase in yield. After 96 hours (reaction 5, **Table 1**) an increased yield of only 3%, to 51% was observed. Sufficient amounts of the desired product had then been synthesized and further optimizations for this reaction were not performed. Future work could include the exploration of other solvents such as 1,4-dioxane at higher temperatures.

Table 1: *Reaction conditions for achieving the optimization of production of compound* ***27***

Reaction number	Time in hours	Temperature in °C	Solvent used	Percentage yield In %
1	24	80	EtOH	10
2	24	66	THF	32
3	48	25	EtOH	5
4	72	66	THF	48
5	**96**	**66**	**THF**	**51**

Synthesis of ethyl 3-(benzo*[d][1,3]*dioxol-5-ylamino) propanoate (**28**) was accomplished through a Michael addition (1,4-conjugate addition) between ethyl acrylate and 3,4-(methylenedioxy)aniline (**SM1**). Initially the synthesis proved challenging, with several catalysts having been employed (reactions 1 and 2, **Table 2**). These reactions were based on using Cu(I), and Fe(II) complex formation.[46] However, the use of these catalysts proved unsuccessful in providing significant yields. This was likely due to the auto-polymerization of ethyl acrylate in the absence of a radical scavenger; however, this was not investigated. At first compound **28** was synthesized by the author, through a neat reaction between the two compounds under microwave irradiation (reactions 3 and 4, **Table 2**).[47,48] The use of the neat reaction, though unusual, has been used in reactions between amines and α,β-conjugated carbonyl compounds and is discussed in-depth by Ranu *et al.*,[47] due to the environmentally friendly nature of the reaction when applied. The solvent-free methods allowed for the formation of the desired final product **28**, with yields ranging from 17−26% (**Table 2** reactions 4 and 5). The use of microwave heating in 1,4-conjugate addition is discussed by Escalante *et al.*,[48] in which primary amines were successfully reacted with methyl acrylates in methanol. It is well known that microwave irradiation (MWI) offers various benefits in organic synthesis, such as faster reaction times and accommodating milder reaction conditions than conventional heating methods.[49] The S_N2 strategy was also employed using ethyl-2-bromo-ethanoate however the final yield of **28** was found to be only 22%, as shown in **Table 2**, reaction 8, the low yield can be attributed to bis-alkylation of the product, however this was not thoroughly investigated.

Product **28** was best produced under conventional heating at 60 °C for 72 hours, without solvent, following the method proposed by Ranu, Dey, and Hajra in 2002 (see **Scheme 3**).[47,50] This yielded the product in 56% yield (**Table 2** reaction 7). The structure was confirmed using analytical techniques such as ^{1}H NMR spectroscopic analysis, with the appearance of peaks at 4.15, 3.37, 2.58 and 1.26 ppm, accounting for the ester moiety which corresponded well with the literature.[51] In addition, mass spectroscopy showed an *m/z* value of 238.1071, where the expected exact mass (calculated M+H) for the compound is 238.1079.



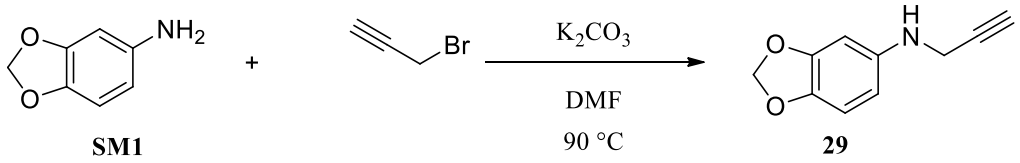
***Scheme 3**: Synthesis of ethyl ester functionalized aniline*

Table 2:** Reaction conditions for achieving the optimization of production of compound **28

Reaction number	Time in hours	Temperature in °C	Solvent	Catalyst	Percentage yield in %
1	40	40	DCM	$FeCl_2$ & NaH	0
2	96	25	Toluene	Cu(I)Cl, PPh_3, KO*t*Bu	0
3	1	98	Ethylene glycol	Microwave Irradiation	2
4	3	80	neat	Microwave Irradiation	17
5	24	25	None	None	26
6	27	58	None	None	22
7	**72**	**60**	**None**	**None**	**56**
8	96	66	THF	None	22

The addition of the alkynyl functional group to form compound **29** was achieved with the best yield of 72% by reacting propargyl bromide and 3,4-(methylenedioxy)aniline in DMF with K_2CO_3 at a temperature of 90 °C for 2 hours (**Table 3** entry 3, **Scheme 4**). Table 3 details the optimizations performed

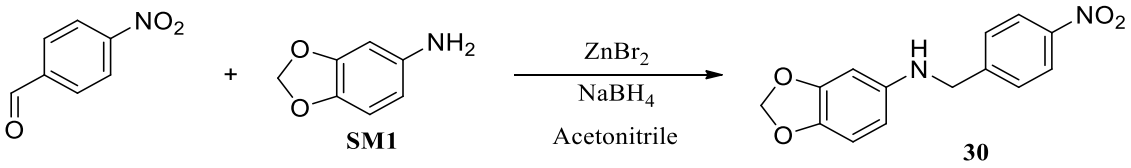
to obtain product **29** with a maximum yield of 72%. HRMS showed the presence of peaks at *m/z* 176.706 where the calculated expected M+H value was 176.0712. The ^{1}H NMR spectrum of the minor product (see Supplementary information) showed peak integrations twice that of the major product for the propargyl moiety at 2.22 ppm and 3.87 ppm, indicating the presence of a tertiary amine due to bis-alkylation of **SM1**, accounting for the lost yield (20% approximate yield of minor product). Acetonitrile was attempted as a solvent, however, this produced significantly lower yields (15 and 44%), under the same conditions in DMF (**Table 3**, entries 1 and 4).



***Scheme 4**: Synthesis of propyne functionalized aniline*

Table 3**: Reaction conditions for achieving the optimization of production of compound **29

Reaction number	Time in hours	Temperature in °C	Solvent	Percentage yield in %
1	0,5	82	Acetonitrile	15
2	2	25	DMF	18
3	**2**	**90**	**DMF**	**72**
4	2	90	Acetonitrile	44
5	6	25	DMF	49
6	24	25	THF	24



***Scheme 5**: Synthesis of para-nitrobenzene functionalized aniline (**30**)*

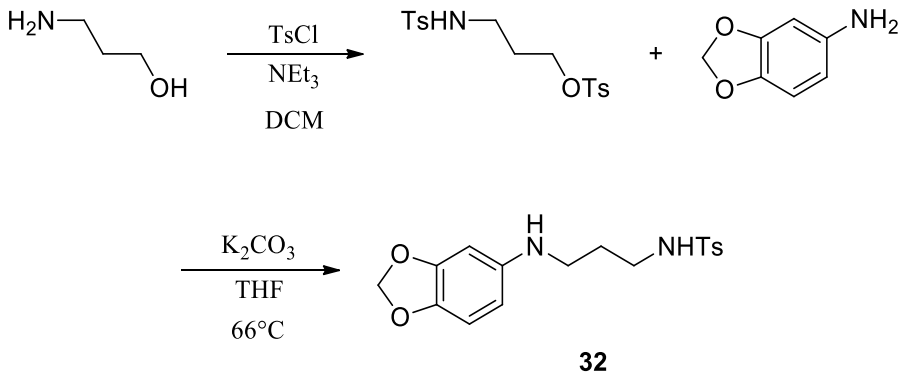
Compounds **30** (*para*-nitrobenzene functionalized, **Scheme 5**) and **31** (*meta*-nitrobenzene functionalized) were successfully synthesized in significant yields using reductive amination between 3,4-(methylenedioxy)aniline and a nitro benzaldehyde. Products **30** and **31** were optimally produced in 61% and 70% yield respectively (reaction 9, **Table 4**). The synthesis of compound **30**, was also achieved using

an S_N2 approach. 3,4-(Methylenedioxy)aniline (**SM1**) and 4-nitrobenzyl bromide were reacted, and produced the desired product (reactions 3,4 and 5, **Table 4**). The yields obtained using this approach were significantly lower, with the best being 30%. It is proposed that this is likely due to the formation of the bis-alkylated product as suggested by ^{1}H NMR spectroscopy (see Supplementary information).

The ^{1}H NMR spectra showed the appearance of peaks in the aromatic range, as well as at 4.40 ppm for **30** and 4.38 ppm for **31**, in addition IR spectroscopy also showed peaks in the 1450-1550 cm^{-1} range indicative of the aromatic NO bond. Formation of products **30** and **31** was accomplished in the highest yields after 36 hours by *in situ* formation of selective reducing agent $Zn(BH_4)_2$ using $ZnBr_2$ and $NaBH_4$ in acetonitrile. THF was also used as solvent but resulted in significantly lower yields for **30** (16%, reaction 8, **Table 4**). Other methods of reductive amination, such as using only $NaBH_4$ or zinc powder and KOH were also attempted. The yields for these were significantly lower (all below 30% yield, reactions 6 and 7, **Table 4**).[52,53]

Table 4**: Optimization of the synthesis of compound **30** and applied to the synthesis of **31

Reaction number	Time in hours	Temperature in °C	Solvent	Reagent	Percentage yield in %
1	24	100	Acetonitrile	K_2CO_3	0
2	24	65	THF	K_2CO_3	0
3	6	100	Toluene	K_2CO_3	5
4	24	65	Acetonitrile	K_2CO_3	10
5	24	100	Toluene	K_2CO_3	30
6	24	25	H_2O:MeOH	KOH, Zinc powder	15
7	24	25	THF	$NaBH_4$	28
8	36	25	THF	$ZnBr_2$, $NaBH_4$	16
9	**36**	**25**	**Acetonitrile**	**$ZnBr_2$, $NaBH_4$**	**61**



***Scheme 6**: The synthesis of product **32** using di-tosylated propanolamine.*

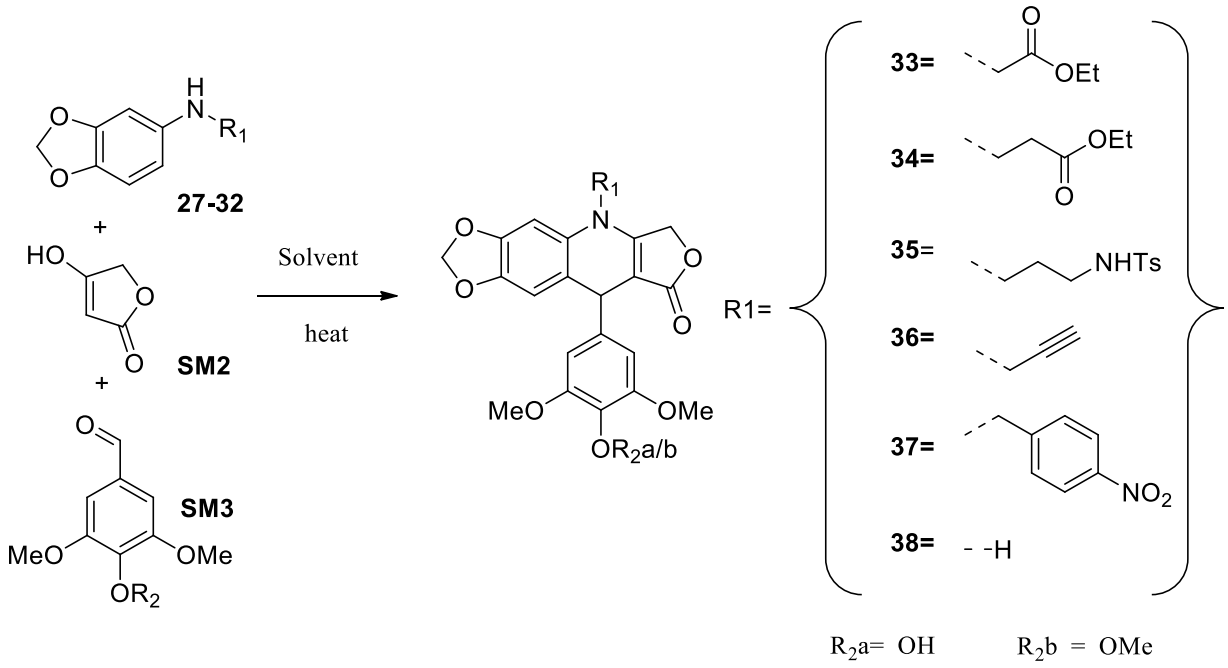
The attempted synthesis of compound **32** was done over two steps (see **Scheme 6**), with the goal that the final MCR product would then be deprotected and used in amide, amino acid or other nucleophilic couplings. The first step was the synthesis of 3-(4-methylphenylsulfonamido) propyl 4-methylbenzenesulfonate in 22% yield from propanolamine. This was accomplished by reacting two equivalents of tosyl chloride with propanolamine in DCM with triethylamine as base. The presence of di-tosylated propanolamine was confirmed by HRMS due to the existence of peaks at *m/z* 383.0932, where the calculated *m/z* for M+H is 384.3909. The ^{1}H NMR spectrum corresponded with values given in literature.[54] Cherepakhin *et al*. mention that the formation of the di-tosylated propanolamine can result in a ring closure reaction to form a 4-membered ring. Fortunately, this ring is still susceptible to ring opening through an S_N2 reaction, allowing the formation of the desired tosylated product.

The product was then reacted with 3,4-(methylenedioxy) aniline and potassium carbonate in DCM at room temperature for 12 hours to yield product **32** with a disappointing 8% overall yield. A TLC of the crude product showed the presence of a significant number of unidentified products. Although product **32** could not be fully characterized nevertheless used in the subsequent reaction.

2.2.2 Synthesis of 4-aza-podophyllotoxin analogues for Library 1

Upon successful synthesis of the *N*-functionalized anilines (**27-32**), synthesis of compounds **33-39** was attempted using the MCR procedure published by Giorgi-Renault *et al*. (see **Scheme 7**).[37] This involved reacting an *N*-functionalized aniline with tetronic acid and a functionalized benzaldehyde in ethanol. In this case, *N*-functionalized 3,4-(methylenedioxy)aniline, tetronic acid and either syringaldehyde (for all **A** labeled compounds) or 1,2,3-trimethoxybenzaldahyde (used for compounds **B**). To accommodate microwave irradiation (MWI), for faster and shorter reaction times, ethylene glycol was used as a solvent instead of ethanol. Typically ethanol is used (boiling point 78 ^{o}C), as proposed by Giorgi-Renault *et al*.[55] Ethyleneglycol was used for the microwave reactions due to its higher boiling point (197.3 ^{o}C) to prevent pressure buildup. In addition 4-chloroaniline was used as a sacrificial aniline, as published by Jeedimalla *et al.*[43] 4-chloroaniline has a much lower electron-donating ability when compared to 3,4-(methylenedioxy)aniline (**SM1**) and during the reaction 4-chloroaniline was replaced by the required functionalized aniline after Knoevenagel condensation (see **22** in **Chapter 1**, **Scheme 1**, for more details about this mechanism).

As has been discussed in **Chapter 1**, during the formation of the 4-aza-podophylltoxin analogues, there is no control of stereochemical elements in the C ring, resulting in two enantiomers being formed (relative to the C1 position). Recent research has been done into increasing enantiomeric selectivity during the MCR; however, little to no enantiomeric excess has been observed (**24** and **25**, **Scheme 1**, **Chapter 1**),[43] therefore, the general biological data for these compounds represent that of a racemic mixture.[8] Though the enantiomers are not expected to share identical biological activity the initial testing was performed on the enantiomeric mixture. The idea was that if a racemic mixture was found to be significantly active, further efforts could be focused on testing of the individual enantiomers. [1]



Scheme 7: *The compounds attempted for the synthesis of Library 1, for biological testing*

2.2.2.1 ***Compound 33: 4N-analogue modified by the addition of methyl ethanoate***

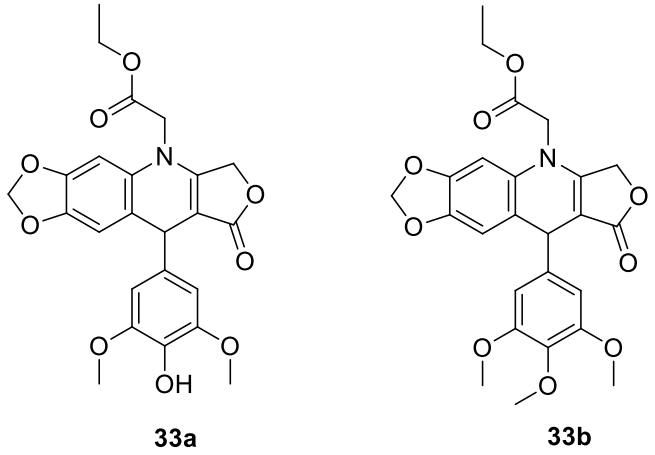


Figure 10: *Structures of methyl ester4N-functionalised compounds **33a** and **33b***

Synthesis of compound **33** using MWI and sacrificial aniline 4-chloroaniline led to higher yields (44% for compound **33a**, *m/z*: 470.1446, M+H calculated to be 470.1451 *m/z*) when compared to the yield of the catalyst-free reaction (26% for compound **33a**). Conventional heating of the reaction led to a significantly lower yield of <5% (for compound **33a**), when run for the same time. The successful synthesis of compounds **33a** and **33b**, (35% yield for **33b**, *m/z*: 484.1602, calculated to be 484.1608 *m/z*) was achieved

and confirmed by IR, MS and NMR spectroscopic analysis. The ^{1}H NMR spectroscopic analysis of **33a** showed a triplet peak at 1.24 ppm, indicative of the methyl carbons on the ester moiety (from **27**), as well as a peak at 3.13 ppm, which indicated the presence of the methoxy functional groups on syringaldehyde (**SM3**). A signal at 4.78 ppm was also observed, corresponding to the D ring CH_2 group (from tetronic acid, **SM2**). Similar peaks were observed for **33b**, though integration of peaks at 3.13 showed 9 protons corresponding to 1,2,3-trimethoxybenzaldehyde (**SM3**).

Like compound **33a**, **33b** was successfully synthesized in 35% yield using the method proposed for **33a.** Of note was that when halving reaction times to only 1 hour under MWI, only 17% yield was achieved for compound **33b**.

2.2.2.2 *Compound 34: 4N-analogue modified by the addition of ethyl ethanoate*

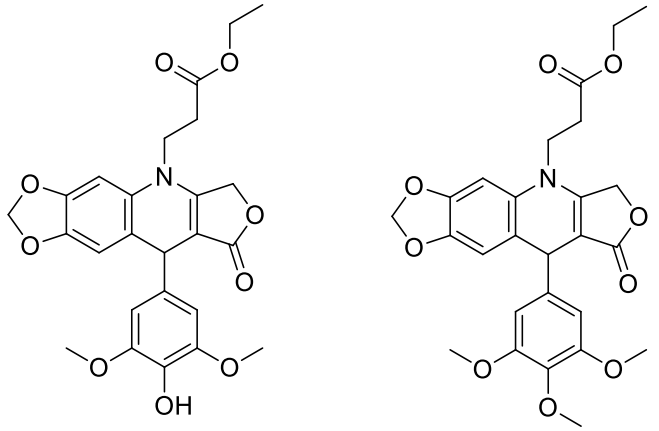


Figure 11**: Structures of ethyl ester 4N-functionalized compounds **34a** and **34b

Compound **34a** was only achieved in 8% yield (HRMS: calculated (M+H) 484.16 *m/z*, found peak at 484.1596 *m/z*) using the method used for **33a** and **33b**. This led to experimentation with lower temperatures and shortened reaction times (110°C for 35 minutes). However, the largest yield obtained, 8%, was still obtained under MWI, with the sacrificial aniline, at 180 °C for 2 hours. In addition, compound **34b** (HRMS: calculated (M+H) 496.1608; found peak at 496.1609 *m/z*.) was obtained in a similarly disappointing 14% yield under the same conditions. We propose that the low yields obtained can be attributed to reactions with the ester moiety, either through the formation of an amide to replace the ester

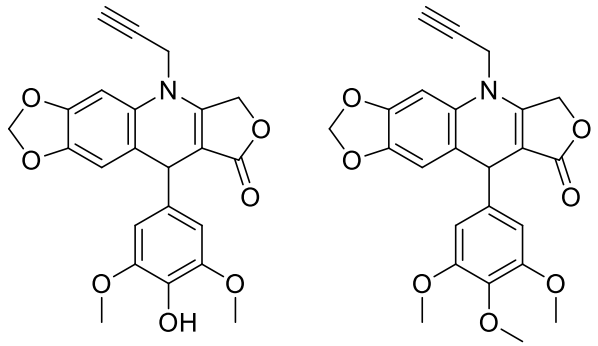
or through unwanted ring closure, through the Skraup reaction, however this remains speculation.[56] The longer chain ester is also more reactive than its short chain counterpart (**27** and **33a/b**). This is due to its ability to more easily form the enolate isomer due to the lower pKa of the proton's alpha to the carbonyl moiety, and increased stability due to the inductive effect.[57]

The increased potential to form minor products may have impacted the yields obtained. Similar peaks were observed in the ^{1}H NMR spectrum as for those of **33a/b**; however, extra peaks were observed in the 1.28-1.13 ppm range, for **34b,** corresponding to the peaks expected for the longer carbon chain in the 4*N* position. It was suspected that the missing peaks in the ^{1}H NMR spectrum for **34a** was due to the previously proposed enolization of the ester functional group.

2.2.2.3 *Compound 35: 4-N analogue modified with tosyl protected amine*

Synthesis of compound **35a/b** was unfortunately not achieved. ^{1}H NMR spectroscopy showed the presence of the peaks corresponding to the tosyl protected amine; however, mass spectroscopy showed masses inconsistent with the expected product. It is proposed that the tosylated amine was still reactive enough to inhibit the formation of the desired product during the multicomponent reaction, instead forming undesired side products. Mass spectroscopy also indicated the formation of minor products with compound **35a** showing a *m/z* of 585 and 501, where the calculated expected M+H value was 594 *m/z*. Compound **35b** showed the appearance of peaks at 455 and 587 *m/z*, while the calculated expected value was 609 *m/z*. This indicates that the final desired ring closure did not take place. Further optimization of the synthesis of compound **32** will need to be performed before further attempts at synthesis can be made. It was hoped that if synthesis was successful the amine could be deprotected under mild conditions and low temperatures and then used as a nucleophile in other coupling reactions.

2.2.2.4 *Compound 36-4N analogue modified with propyne moiety*



36a **36b**

Figure 12**: Structures of propyne 4N-functionlized compounds **36a** and **36b

Compounds **36a** and **36b** were produced in 32% and 66% yields respectively. The synthesis of compound **36a** was attempted multiple times, with the best yield of 32% being obtained under MWI at 110°C for 7 minutes and 30 seconds. Mass spectroscopy of **36a** revealed a *m/z* value of 422.1238 *m/z* while the calculated value for **36a** was at 422.1240 *m/z.* On the other hand, **36b** was obtained after 10 minutes at 150°C under MWI. Mass spectroscopy found the relevant peak corresponding to **36b** at 436.1382 *m/z* which correlates well with the expected calculated value (M+H), which was 436.1396 *m/z.* The reasons for the differences in yield remain unclear and have not been explored further in this research project. Products **36a** and 3**6b** both showed the presence of peaks corresponding to the propargyl-moiety between 3.80 and 4.20 ppm in the ^{1}H NMR spectrum. IR spectroscopy also showed the appearance of a broad peak in the 1250-1450 cm^{-1} range, indicative of the alkyne functional group.

2.2.2.5 *Compound 37: 4N-analogue modified with para-nitrobenzene*

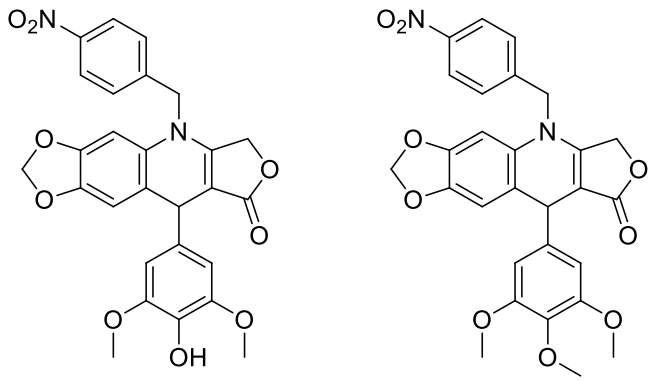


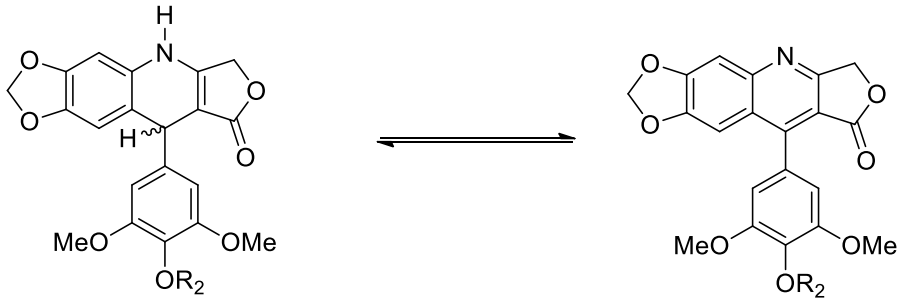
Figure 13**: Structures of para-nitrobenzene functionalized compounds **37a** and **37b

Synthesis of compounds **37a** and **37b** were achieved in 61% and 15% yield respectively. Upon heating under MWI, compounds **37a** and **37b** were not observed, however, under conventional heating to 198°C for 30s both products were obtained. It is proposed that the electron-withdrawing effect from the nitro group on compound **30** may have influenced the reaction, by improving the reactivity of the amine. For compound **37a** a calculated mass (M+H) of 519.1404 *m/z* was expected, while experimentally using HRMS a value of 519.1406 *m/z* was obtained. For compound **37b** the HRMS expected a calculated *m/z* (M+H) of 533.1560, a molecular peak was observed at 533.1594 *m/z*. IR spectroscopy also showed peaks in the 1450-1550 cm^{-1} range, indicative of the aromatic NO_2 group. Proton NMR spectroscopy also showed the appearance of new peaks in the aromatic region when compared to **33a** and **33b**.

2.2.2.6 *Compound 38: unfunctionalized 4N-aza-podophyllotin analogues*

Both compounds **38a** and **38b** were obtained after 10 minutes under MWI at 150 °C; compound **38a** finally, with 43% yield and compound **38b** with a 28% yield. Both compounds **38a** and **38b** showed little formation under higher temperatures or longer reaction times; instead forming what was suspected to be mixtures of polymers. No additional analysis was performed on this product, and it was not further investigated. When attempted at lower temperatures no yield was observed for both **38a** and **38b**. The mass spectrum for **38a** showed the presence of a large peak at 382.0922 *m/z* (M+H) and a smaller signal at

384.1069, compared to the calculated expected mass which should be 384.0888 *m/z*. This indicated the molecular formula to be $C_{20}H_{16}NO_7$; however, this and NMR spectroscopy did not correlate with literature reported values. The ^{1}H NMR spectrum obtained indicated the absence of two peaks, correlating with the missing hydrogen atoms expected for an oxidation product.[37] They both indicated the loss of 2 protons relative to the initially reported structure, most likely due to the oxidation of the product (see **Figure 14**). The mass spectrum for **38b** shows peaks for $C_{21}H_{20}NO_7$, 398.1236 *m/z*, while the calculated mass (M+H) was 398.1240 *m/z*, which is consistent with literature.[37] Despite **38a** not corresponding to literature reported values, the biological testing of compounds **38a** and **38b** correlated well with the expected values and showed potencies higher than the other compounds synthesized in Library 1.



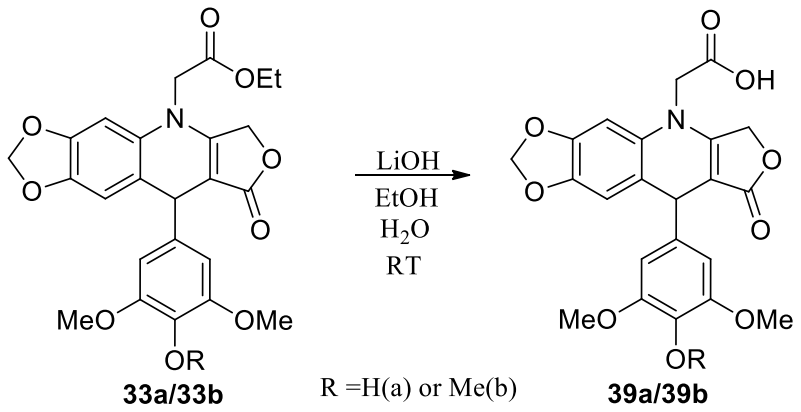
R_2= -OH(**a**) Or OMe(**b**)

Figure 14**: Proposed oxidation of compounds **38a** and **38b

2.2.2.7 *Hydrolysis of compounds 33a and 33b*

In order to test the availability of the synthesized esters (**33a** and **33b**) for further modification, without the disruption of the overall structure, hydrolysis of the desired esters was attempted under basic conditions. This was chosen because of the sensitive nature of the lactone ring (D ring, **Figure 7**).[36]

The successful synthesis of compounds **33a** and **33b** allowed for the attempted hydrolysis of the compounds, to produce **39a** and **39b**. Hydrolysis was attempted by reacting 3**3a** and **33b** separately, with lithium hydroxide and H_2O in ethanol overnight, at room temperature (see **Scheme 8**).



Scheme 8: *Hydrolysis of esters **33a** and **33b** to carboxylic acids **39a** and **39b** with base.*

Both MS and IR as well as ^{1}H NMR spectroscopy show the formation of the hydrolyzed forms of **33a** and **33b**, i.e. **39a** and **39b**. LCMS, however, showed <50% purity of the compounds. Unfortunately attempts at separation failed. Even after ethanol recrystallization or flash column chromatography TLC analysis showed the presence of starting material **33a** or **33b**. For the latter method the starting materials appeared to elute at similar times to **39a** or **39b.** It is suspected that ring opening occurred at D ring due to LiOH resulting in minor products (**see discussion in Chapter 1, Figure 6**).[58] However for the most part, these conditions were mild enough to hydrolyze the ester without excessive hydrolysis of the lactone group present on the molecule.[59] To be clear, the desired reaction took place, despite the formation of small amounts of the minor product. Yields of the crude mixtures of 99% (**39a** in 68% purity) and 43% (**39b**, crude <50% purity) were achieved respectively.

^{1}H NMR spectroscopic analysis suggested conversion to the acidic form of the 4-aza-podophyllotoxin (**39a** and **39b)** was successful; despite the presence of some starting material. LCMS analysis showed the presence of both **33a/b** and **39a/b** in the crude product obtained. Further purification is however required. This could be accomplished through HPLC, as flash column chromatography of these compounds has not been sufficient for purification.

In terms of spectral analysis, the crude mixture containing compound **39a** showed a major peak present at *m/z,* 442.1142, where the calculated expected mass (M+H) was 442.1138 *m/z*. Compound **39b** showed a *m/z* peak at 456.1276 (corresponding to the calculated expected value of.456.1276 *m/z*). Staining with Bromocresol green, as well as IR spectroscopy confirmed the presence of the acidic form of the desired compounds (**39a** and **39b**). IR spectroscopy showed the appearance of broad, intense bands in the OH region (2500-3700) of the spectra, that was not apparent in the spectra for compounds **33a** and **33b**.[60]

2.3 Synthesis of Library 2: 4-aza-podophyllotoxin analogues synthesized through copper mediated alkyne azide cycloadditions

With the information gathered from Library 1, as well as the biological results (discussed in **Chapter 4**), the synthesis of Library 2 proceeded with the intent to further modify products **36a** and **36b**. The choice was motivated by both their significant biological activity (IC_{50} = <275 nM), [1] as well as their ability to be further modified in the absence of acidic conditions, specifically their potential to undergo 'Click' chemistry.[61] The use of a copper-mediated azide alkyne cycloaddition (CuAAC), was further motivated by its potential to introduce more nitrogen atoms into the analogue scaffold. It should also be noted that GL331 (**14**) has a nitrogen atom in the α position when compared to the podophyllotoxin scaffold. It is proposed that this may improve its selectivity to cancer cells.[1]

The incorporation of a triazole ring may further improve selectivity, as well as biological activity, despite the increased molecular weight. This increase in weight may prevent the crossing of the analogue over the cellular membrane;[20] however, the cancer cells increased need for nitrogen for metabolic activity may overcome this. Copper-mediated click chemistry also shows great selectivity, specifically in generating

the 1,4-triazole, instead of the 1,5 triazole.[62] The synthesis of azides, shown in Figure 15, was attempted in preparation for the synthesis of the compounds in Library 2,with 4 out of the 5 syntheses being successful.

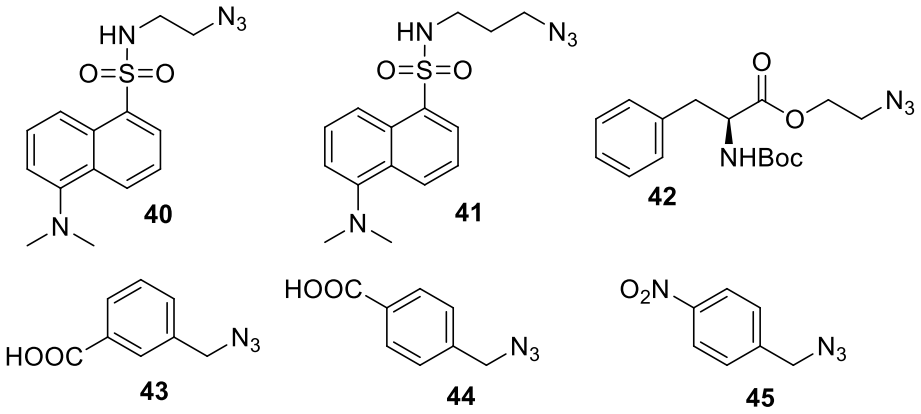


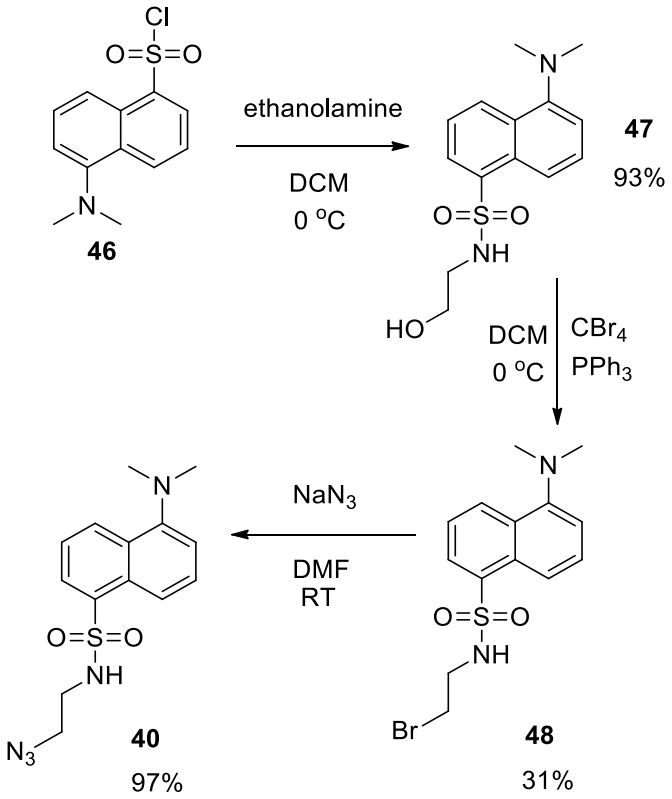
Figure 15: *Desired azides for use in click reactions*

Each compound was chosen for their individual characteristics. Compounds **40** and **41**, were chosen because they can act as fluorescent probes for the observation of the movement of the 4-aza-podophyllotoxin analogues *in vitro*. They were also chosen with different lengths of the carbon chains to determine if the dansyl azide affects biological activity, as well as the steric and free rotating implications of the chain length when in the cell. Compound **42** was picked to determine if the incorporation of amino acids into the cancer cell would improve biological activity. Azides **43** and **44** were chosen to provide a basis of comparison with **45,** as well as for their potential to further form benzoic esters if desired. Compound **45** was selected to compare with the clinically reviewed GL-331 (**14**), as well as with compounds **37a** and **37b**. The following section provides a discussion on the synthesis of the desired azides, in numerical order, and the click reactions performed.

2.3.1 Synthesis of azides 40-45

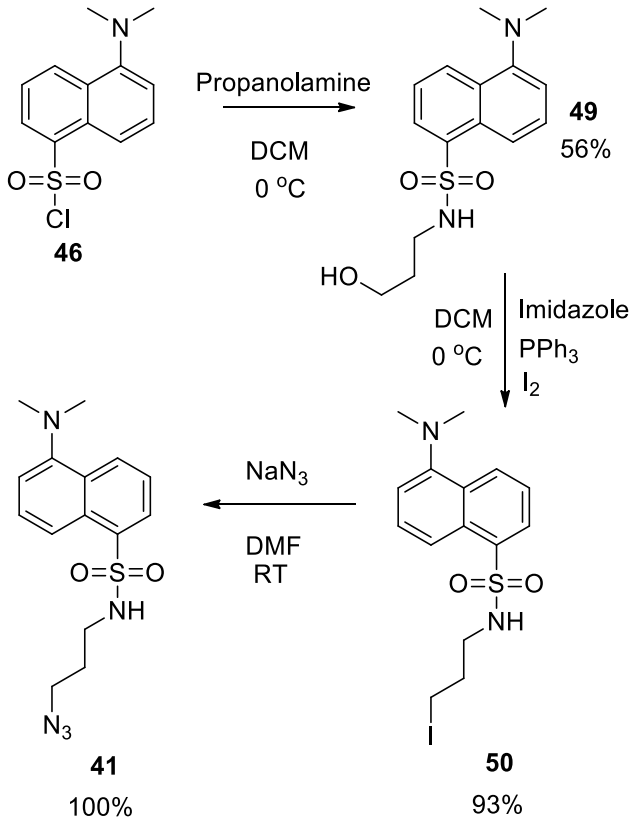
Compound **40** was produced over three steps from commercially available dansyl chloride **46**. This was achieved by first synthesizing compound **47**, then **48**, to finally yield **40**. Compound **47** was synthesized by reacting ethanolamine, commercially available dansyl chloride **46** and triethylamine in DCM at 0°C for 1 hour, yielding **47** in 93%. Compound **48** then had to be synthesized, and was produced in 31% yield using an Appel reaction.[63] This was accomplished using product **47**, tetrabromomethane, triphenyl phosphine and DCM at 0 °C. Product **48** was formed after 2.5 hours, as per TLC, before isolation using flash column chromatography with DCM:methanol as solvent.

Finally, the synthesis of **40** was performed using sodium azide, dansyl ethyl bromide **4**), in DMF at room temperature. Product **40** was obtained after 1 hour in 97% yield from **48**, but with an overall yield of 28% after all three steps. This was confirmed by mass spectroscopy that presented a signal at 320.1173 *m/z* which compared well with the calculated mass of **40** (M+H) 320.1181 *m/z*. Furthermore proton NMR spectroscopy and MS data correlated with literature reported values. [64]



Scheme 9: *The synthesis of **40** from dansyl chloride **46***

The synthesis of compound **41** was attempted in a very similar fashion. Using propanolamine, triethylamine, and dansyl chloride in DCM at 0°C, compound **49** was synthesized in 56% yield after 1 hour using the S_N2 approach. The low yield might be consequence of the reaction of the alcohol functional group with the sulfonyl chloride instead of the amine, despite the greater stability of the sulfonamide functional group over the sulphonyl acid ester. It is suspected that this occurred despite the greater nucleophilicity of the nitrogen lone pair over those of oxygen. The difference in yield for **47** compared to **48** is unfortunately unclear. A modified Appel reaction was employed to convert the primary alcohol on **49** to the iodo moiety on **50**. This was achieved in 93% yield using triphenyl phosphine, imidazole and iodine in DCM at 0°C for 1hour, this method will be used for future synthesis of compound **48** due to its improved yields when compared to the synthesis of **50**. The product, **50**, was then converted into an azide using sodium azide as a nucleophile in DMF at room temperature. This yielded product **41** with 100% yield after 5 hours, but with an overall yield of 51% over all three steps. IR spectroscopic data showed the presence of peaks in the 2050-2100 cm^{-1} range indicative of an azide. This product was also confirmed by comparison of the ^1H NMR spectrum with that reported in lterature.[64]



Scheme 10: *Synthesis of dansyl azide* ***41*** *from dansyl chloride* ***46****.*

The attempted synthesis of Boc-protected phenyl alanine azide, **42**, was performed in two parts. First the synthesis to produce the Boc-protected L-phenylalanine using L-phenylalanine, di-*tert*-butyl dicarbonate, and sodium hydroxide in water and THF was performed. This achieved the Boc protected amino acid in 87% yield. Mass spectroscopy was used to determine the mass-to-charge ratio of the intermediate. The expected mass (M+H) was calculated to be 266.1392 *m/z*, which correlated well with the signal in the mass spectrum at 266.1385 *m/z*. The synthesis of **42** was attempted reacting 2-azidoethyl methyl sulfonate, sodium hydride, the previously made Boc-protected amino acid and DMF at room temperature. However, the L-phenylalanine Boc protected azide was not produced after 24 hours. The absence of the final product was confirmed using the ^{1}H NMR and mass spectrum, which did not correlate with literature data.[65]

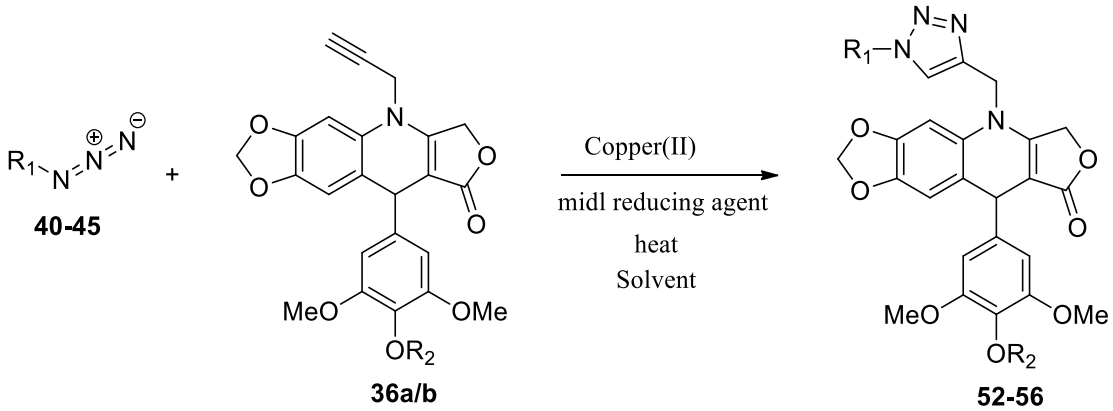
Compounds **43** and **44** were produced by a member of the synthetic chemistry group at Stellenbosch University (Mr. M.G. Botes) and utilized as is.

Finally, product **45** was synthesized by reacting sodium azide in DMF with 4-nitrobenzyl bromide for 5 hours at room temperature, producing product **45** in a yield of 95%. This product was confirmed by ^{1}H NMR spectroscopy which corresponded exactly to literature values.[66] At the time of writing further confirmation by mass spectroscopy was awaited.

2.3.2 **Click reactions**

Literature discussing copper-mediated azide-alkyne cycloadditions on podophyllotoxin scaffolds typically reports the use of azides in the α or β position to the C4 carbon, and reacting them with functionalized alkynes.[61] Due to the restriction of using a nitrogen atom in the C4 position, as seen in 4-aza-podophylltoxin analogues, it was necessary to take a unique approach resulting in the use of an alkyne in the 4*N*-position. This approach was not entirely successful. Although four of the ten desired compounds were synthesized (see **Figure 16**), synthesis required extensive optimization and extensive purification.

In order to synthesize compounds **51-56** from products **36a** and **36b**, as shown in **Figure 12**, several methods were employed, based on the work of Sharpless *et al.*(see **Scheme 11**).[67] For all syntheses copper acetate and copper sulfate were chosen as the starting point for generating the Cu(I) catalyst *in situ.*[62] They were chosen for their potential to be reduced by weak reducing agents such as sodium L-ascorbate. In principle, click reactions can be performed without the need to employ a copper catalyst. However in efforts to selectively synthesize the 1,4-click products, no catalysis-free click reactions were attempted.[67]



***Scheme 11**: General method of synthesis of alkyne azide cycloaddition products*

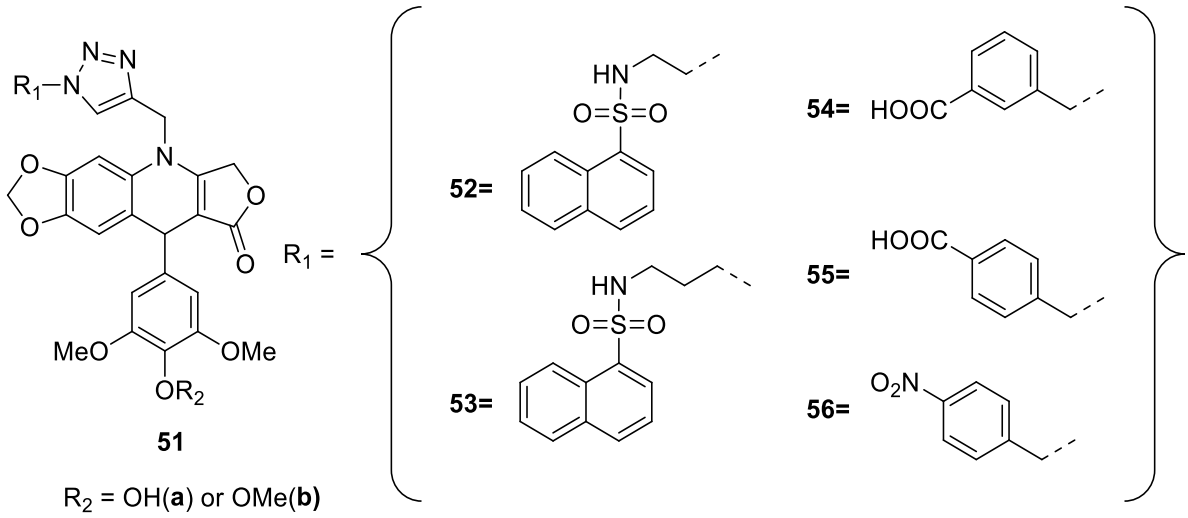
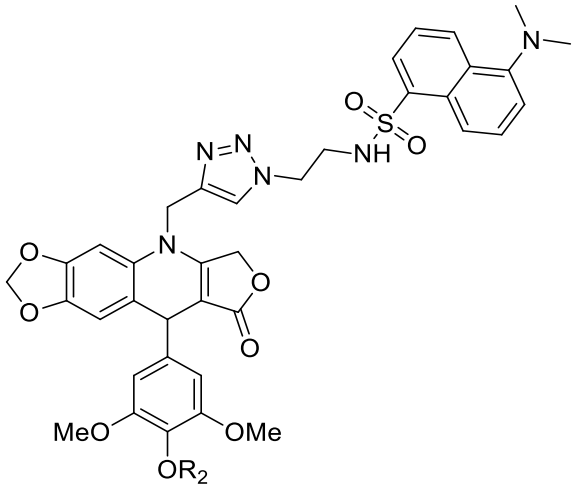


Figure 16: *4-Aza-podophylltoxin analogues attempted through click chemistry of azides* ***40-45*** *(excluding* ***42****) with 4-aza-podophylltoxin derivatives* ***36a*** *an* ***36b****.*

Initially the synthesis of click products **52-56** (**Figure 16**) were attempted by mixing **36a** or **36b** with the corresponding azide, a copper catalyst, and sodium ascorbate in various solvents for several hours. Water was used to force the precipitation of the resultant product. Upon analysis of the residues formed for compounds **53a, 53b, 54b,** and **55b** using ^{1}H NMR spectroscopy, it was concluded that no product formation had occurred. For these compounds only the initial 4-aza-podophylltoxin analogues **36a** and **36b** were recovered and little to no formation of the desired product was observed (<5%) as per the ^{1}H NMR spectra. This may be as a result of solubility issues of compounds **36a** and **36b**. A series of tables can be found in the experimental section (**Tables 10-13**, **Chapter 3**) illustrating the synthetic methods attempted for these compounds. This section below discusses the synthesis, in numerical order, from product **52-55**.



R_2= -OH (**a**) or -OMe (**b**)

52

***Figure 17**: Expected structure of compound **52** synthesized from **36a/b** and Azide **40**.*

Several methods were attempted for the syntheses of compounds **52a** (reactions 1-6 and 13 **in Table 5**) and **52b** (reactions 7-12 and 14 in **Table 5)**. A variety of solvents (DMSO, *t*BuOH:H_2O, DMF, and DCM:H_2O) were used in the synthesis of **52a**, in an attempt to improve yield (see reactions 1,2,3 and 5, **Table 5**). However, after several attempts, little to no final product formation was observed in the ^{1}H NMR spectra. Attempts were then made at altering the Cu(I) catalyst, from copper acetate to copper sulfate. Unfortunately, no change was observed, and yields did not improve. Subsequently the amount of sodium L-ascorbate used and temperature were increased, resulting in a very slight increase of yield (reaction 6, **Table 5**), potentially due to the increased amount of Cu(I) present.[68] The decision was then made to employ the Cu(I) stabilizing ligand THPTA. THPTA effectively increases the concentration of Cu(I) in solution and thus acts as a click accelerator. It is most commonly used in bioconjugation as it can prevent the oxidation of histidine and other molecules sensitive to peroxides and radicals formed from the copper catalyst.[69]

Compound **52a** was then successfully synthesized in 55% crude yield by adding ligand THPTA, using the reaction conditions specified in **Table 5** reaction 13 (indicated with an asterisk (*))[69].The presence of **52a** was proved through the disappearance of alkynyl peak in the ^{1}H NMR spectrum and the appearance of the aromatic, and triazole, peaks corresponding to the fluorescent moiety, **40**. This was verified by the

appearance of a peak at 741.2325 *m/z* in the mass spectrum where the calculated expected value for M+H was 741.2343 *m/z*. Unfortunately, the starting material and a 2M+H peak representing THPTA were also observed in the mass spectrum for the final product. The product therefore requires further purification before it can be sent for biological testing.

The use of THPTA and alteration of other reaction conditions did not increase the yields for product **52b.** This may be due to a difference in solubility between those compounds with the phenolic functional group compared to those with the methoxy functional group in the R_2 position (see **Figure 17**). Although the mass spectrum did show the appearance of a signal at 745.2467 *m/z* which corresponds to the expected calculated mass of 745.2264, the ^{1}H NMR spectrum did not show sufficient presence of the product. Minor peaks were observed in the higher aromatic range (upwards of 7.0 ppm) however those peaks did not offer conclusive evidence for the formation of product **52b.**

Table 5: *Methods attempted for the synthesis of compounds **52a** and **52b**.*

Reaction number	Click Product	Solvent	Time in hours	Temperature	Catalyst(s) (5%)	Amount of sodium L-ascorbate (in eq.)	Yield in %
1	**52a**	DMSO	24	RT	$Cu(OAc)_2$	0.1	<5%
2	**52a**	DMF	24	RT	$Cu(OAc)_2$	0.1	<5%
3	**52a**	*t*-Bu:H_2O	24	RT	$Cu(OAc)_2$	0.1	<5%
4	**52a**	*t*-Bu:H_2O	24	RT	$Cu(SO_4)\cdot 5H_2O$	0.1	<5%
5	**52a**	DCM:H_2O	6	RT	$Cu(SO_4)\cdot 5H_2O$	0,1	<5%
6	**52a**	DMSO	24	60	$Cu(OAc)_2$	0.3	<10%
7	**52b**	DMSO	24	RT	$Cu(OAc)_2$	0.1	<5%
8	**52b**	DMSO	32	RT	$Cu(OAc)_2$	0.1	<5%
9	**52b**	DMSO	12	RT	$Cu(SO_4)\cdot 5H_2O$	0.1	<5%
10	**52b**	DMF	24	RT	$Cu(SO_4)\cdot 5H_2O$	0.1	<5%
11	**52b**	DCM:H_2O	6	RT	$Cu(OAc)_2$	0,1	<5%
12	**52b**	DMSO	24	60	$Cu(OAc)_2$	0.3	<5%
13	**52a***	**DMSO**	**24**	**RT**	**$Cu(SO_4)\cdot 5H_2O$**	**0.2**	**55**
14	**52b***	DMSO	24	RT	$Cu(SO_4)\cdot 5H_2O$	0.2	<5%

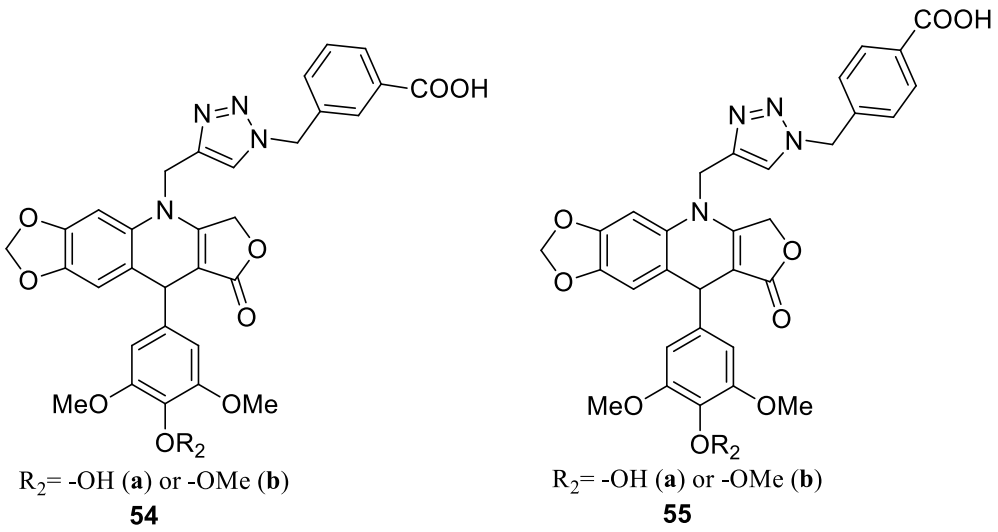


***Figure 18**: Structures of compounds **54** and **55** synthesized from **36a/b** and azides **43** and **44**.*

Product **54a** (see **Figure 18**) was successfully synthesized a yield of 68% with a purity of 96% (LCMS), by heating compound **36a** and azide **43** to 60 °C in the presence of sodium ascorbate (0.2 eq), copper acetate (10% w/w), and DMSO. ^{1}H NMR spectroscopy confirms the formation of the desired product. The ^{1}H NMR spectrum showed the appearance of peaks responsible for the benzylic acid in the 8.4-7.1 ppm range. The mass spectrum also showed a *m/z* value of 599.1768 *m/z*, where the calculated expected value was 599.1778 *m/z*. Compound **54b** was unfortunately not synthesized in sufficient quantities for further analysis. For compounds **54** and **55** the amount of sodium ascorbate was altered first during optimization, as sodium ascorbate helps to buffer the reaction, as a reducing agent, lowering the chance of the benzylic acid affecting the overall podophyllotoxin scaffold.

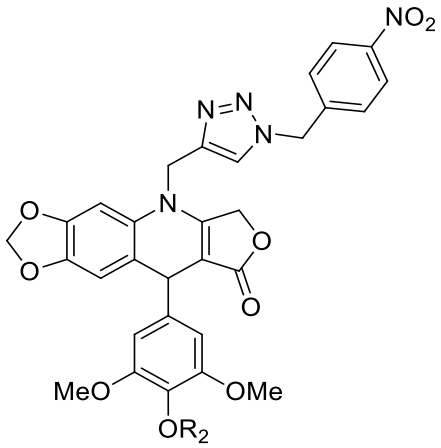
It was initially thought based on ^{1}H NMR analysis, that compound **55a** had been successfully synthesized in 65% yield. However, mass spectroscopy revealed a signal found at 583.1464 *m/z* and 843.2399 *m/z* which did not correspond to the desired product or the starting materials, indicating the potential formation of a currently unidentified product. The difference in mass may indicate a loss of a methyl group (-CH_3). The ^{1}H NMR spectrum revealed peaks at 7.2-8.4 ppm that corresponded with the benzylic acid azide **44** as well as the peaks representing **36a**. The reaction was performed by stirring azide

44, sodium l-ascorbate, copper sulfate, product **36a** and ligand THPTA (**55a***, reaction 7 in **Table 6**) in *tert*-butanol: water (1:1.3) for 24 hours at room temperature.

Although the synthesis of **55b** was attempted, no product formation was observed in either the mass spectrum or the ^{1}H NMR spectrum. Multiple reaction conditions were tried (reactions 4-6, **Table 6**) but no yield of the final product was observed. This may again have been due to a solubility difference between **36a** and **36b.** The mass spectrum of reaction 8 (**Table 6**) revealed that a mixture of starting materials were present in the mass spectrum. Peaks appeared at 871.2703 *m/z*, and 436.1391 *m/z*. This corresponded with THPTA (2M+H) and the starting material, compound **36b**. The reason behind the lowwer yield for those analogues with a methyl functionality on the lower E ring is not yet clear, this may be due to solubility, but further exploration into this matter should be done in the future.

***Table 6**: Synthesis conditions used in the attempted synthesis of **55a** and **55b**.*

Reaction number	Click Product	Solvents	Time	Temperature	Catalyst(s)	Amount of sodium L-ascorbate (in eq.)	Yield in %
1	**55a**	DMSO	24h	RT	$Cu(OAc)_2$	0.1	<2
2	**55a**	*t*-BuOH: H_2O	24h	RT	$Cu(SO_4)\cdot 5H_2O$	0.3	<2
3	**55a**	DMSO	24h	RT	$Cu(OAc)_2$	0.1	<2
4	**55b**	DMSO	24h	40	$Cu(OAc)_2$	0.1	<2
5	**55b**	DMSO	24h	RT	$Cu(OAc)_2$	0.3	<5
6	**55b**	DMSO	24h	60	$Cu(OAc)_2$	0.5	<5
7	**55a***	*t*-BuOH: H_2O	24h	RT	$Cu(SO_4)\cdot 5H_2O$	0.2	65
8	**55b***	*t*-BuOH: H_2O	24h	RT	$Cu(SO_4)\cdot 5H_2O$	0.2	<10



R_2= -OH (**a**) or -OMe (**b**)

56

***Figure 19**: Structure of product **56** from azide **45** and 4-aza-podophylltoixn derivatives **36a/b**.*

Compounds **56a** and **56b** were successfully synthesized. **Table 7** illustrates the improved yields of the synthesis of products **56a** and **56b** 97% and 65%, in DMSO vs that of *t*-BuOH:H_2O. These reactions were performed in the presence of ligand THPTA. The improved yield can be attributed to the solubility of **36a** and **36b** in DMSO when compared to *t*BuOH:H_2O for the compounds **56a** and **56b**. Although the reaction was also attempted with $Cu(OAc)_2$, the yields were significantly lower than those obtained with copper sulfate. The presence of **56a** was confirmed by mass spectroscopy, where the calculated value for M+H was found to be 600.1731 *m/z* while a peak was found at 600.1743 *m/z* in the mass spectrum. The presence of compound **56b** was also confirmed by mass spectroscopy where the expected M+H value of 614.1887 *m/z* while a signal was found at 614.1883 *m/z* in the spectrum. The ^{1}H NMR spectra for both compounds showed sufficient presence of the desired product for conclusive analysis. For both compounds **56a** and **56b**, peaks were observed in the upper aromatic region (7.50 and 8.21 ppm) as well as the peaks typically observed for **36a** and **36b**, without the peaks corresponding to the primary alkynyl and the appearance of the triazole peak.

Table 7**: Synthesis conditions used to produce products **56a** and **56b

Reaction Number	Click Product	Solvents	Time	Temperature	Catalyst(s) (5%)	Amount of sodium L-ascorbate (in eq.)	Yield in %
1	**56a**	*t*-BuOH: H_2O	24h	RT	$Cu(SO_4)\cdot 5H_2O$	0.2	75
2	**56a**	**DMSO**	**24h**	**RT**	**$Cu(SO_4)\cdot 5H_2O$**	**0.2**	**97**
3	**56b**	*t*-BuOH: H_2O	24h	RT	$Cu(SO_4)\cdot 5H_2O$	0.2	65
4	**56b**	**DMSO**	**24h**	**RT**	**$Cu(SO_4)\cdot 5H_2O$**	**0.2**	**78**

Although compounds **52a**, **54a**, **56a** and **56b** showed previously mentioned yields significant enough for further analysis. These compounds were not sufficiently pure for biological testing; however, flash column chromatography of **52a** provided significant data for further purification using a DCM: methanol gradient. It was concluded that further purification with semi-prep HPLC would be required, which was unfortunately not achievable due to time constraints. The irreproducibility of those reactions with **36b** (with the exception of **56b**) is not yet understood and will be a point of investigation for future research.

2.4 Conclusions

Twelve 4-aza-podophylltoxin analogues for Library 1 were successfully synthesized from the 6 *N*-functionalized anilines. Although other anilines were synthesized during this project, they did not yield the desired 4-aza-podophyllotoxin analogues in yields sufficient for analysis. It was found that most products in Library 1 were synthesized in yields below 50%. It was, however, clear that those reactions that made use of microwave irradiation and 4-chloroaniline provided more significant yields. It was also proven that the products from Library 1 can undergo further functionalization without affecting the central 4-aza-podoophylltoxin scaffold. The 4*N*-functionlaised 4-aza-podophylltoxin derivatives from Library 1 were all synthesized in yields sufficient for further analysis. Of these 12 compounds, six contained the phenolic group in the R_2 position and six the methoxy moiety, making them ideal for comparative analysis.

Four compounds were generated for Library 2. These compounds were successfully synthesized but require further purification before biological testing. Of these, three contain the phenolic moiety in the R_2 position and one contains the methoxy moiety in the R_2 position. One analogue was successfully coupled to a fluorescent marker, making it ideal for further biological testing. It was observed that those click reactions which employed THPTA as a ligand performed significantly better than those that did not.

Chapter 3: Experimental methods

The main method of synthesis applied in this research was the multicomponent reaction developed by Giorgi Renault *et al.* in 2010 (as discussed in the literature review).[70] This process, which has been optimized and altered by various groups, remains a key method of synthesis.[1] The current most accepted mechanistic route is discussed by Jeedimalla *et al*. This group also inspired the use of sacrificial aniline, *para*-chloroaniline, in this project.[43]

This chapter will begin by presenting the syntheses undertaken. The synthesis of Library 1 is then described, starting with the synthesis of *N*-functionalized anilines, followed by the synthesis of 4*N*-functionlized-aza-podophyllotoxins, and finally the synthesis of modified compounds from Library 1. Library 2 is then discussed. Library 2 was synthesized from products **36a/b** from Library 1, and a set of azide compounds. The section discussing Library 2 begins with the synthesis of the azides and ends with the 4-aza-podophyllotoxin click reaction products.

3.1 General information regarding experimental procedures

3.1.1.1 *Chemicals and solvents*

The chemicals used in this study were purchased from Sigma Aldrich and Merck, or were sourced from the De Beers Building chemical stores. Ethyl acetate, DCM and hexane were distilled open to the atmosphere before use and were used for TLC plate separation and column chromatography. The solvents used for reactions were distilled under inert conditions over a suitable drying agent: CH_2Cl_2 and acetonitrile over calcium hydride, toluene and tetrahydrofuran (THF) over sodium (benzophenone as indicator for the THF still). These solvents were also stored over 4 Å molecular sieves when distilled.

3.1.2 Laboratory equipment and consumables

Reactions were heated in oil baths using either silicon oil or paraffin oil, unless otherwise specified, using magnetic heater stirrers equipped with temperature probes. Solvents were removed under reduced pressure using Büchi RII Rotovapor rotary evaporators with Büchi V-700 Vacuum Pumps, equipped with V-850 Vacuum controllers, and the final trace amounts of solvents were removed by means of an Edwards high vacuum pump, capable of sustaining a vacuum of 0.08 mmHg. Glassware was dried in an oven at a

temperature of 110 °C. Compounds were stored in Bosch refrigerators and freezers, which were kept at 2 °C and -22 °C respectively. Machery-Nagel ALUGRAM® Xtra SIL G UV254 aluminium sheets were used for thin layer chromatography (TLC) analyses and 254 nm and 365 nm UV lights used for visualization. Various stains were used for the permanent development of TLC plates, including ninhydrin, potassium permanganate, cerium ammonium molybdate, bromocresol green and *p*-anisaldehyde. Iodine was used as a reversible stain.

Column chromatography was done with Merck silica gel (particle size range of 0.063-0.200 mm with 60 Å pores). Auto-column chromatography was performed using a combiFlash®R_f 150, made by TYLEDYNE ISCO. The microwave apparatus used was a CEM Focused Microwave™ Synthesis System, Discover ®SP.

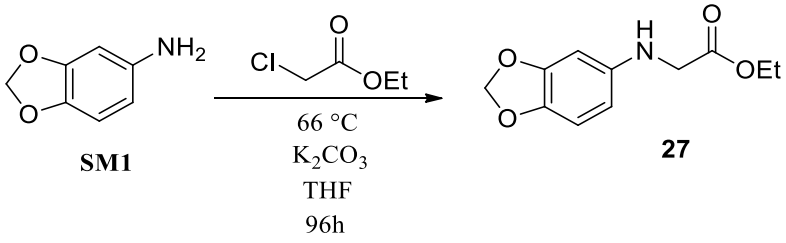
Melting points were obtained using a Gallenkamp Melting Point Apparatus. Infrared spectra were obtained using a Nexus Thermo-Nicolet FT-IR instrument using the ATR function. High resolution mass spectrometry was performed by the CAF (Central Analytical Facility) at Stellenbosch University using either a Waters API Q-TOF Ultima spectrometer or introduced via the ASAP probe using the Waters Synapt G2 spectrometer. Routine mass spectroscopy was performed using a Waters API Quattro Micro spectrometer. In all cases ESI+ was used as an ionisation method. EPR spectra were recorded at 298 K with a Bruker EMX_{plus} X-band EPR spectrometer (8-inch ER 072 magnet, 2.7 kW power supply, EMX-m40X microwave bridge operating from 9.3 to 9.9 GHz), and a high sensitivity continuous-wave resonator.

Samples were polycrystalline solids and dilute solutions of arbitrary concentration in dry dichloromethane or acetone in an otherwise ambient atmosphere. All chromatography was performed using either petroleum ether, hexane, ethyl acetate, methanol and dichloromethane (or a combination of these). Thin layer chromatography (TLC) was carried out on aluminum-backed Merck silica gel 60 F254 plates.

3.2 Library 1: 4-aza-podophylltoxin analogues from *N*-functionalized anilines

3.2.1 Synthesis of *N*-functionalized anilines

3.2.1.1 *Synthesis of Ethyl benzo[d][1,3]dioxol-5-ylglycinate (27)*



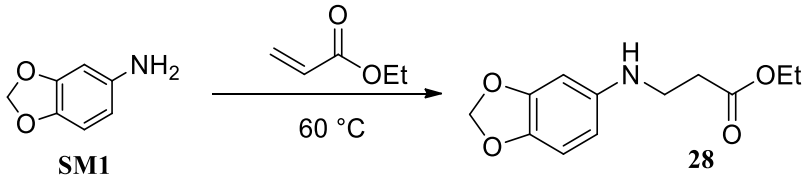
***Scheme 12**: synthesis of methyl ester functionalized aniline (**27**)*

3,4-(Methylenedioxy)aniline **SM1** (0.10 g, 1.0 eq, 0.73 mmol) was added to a solution of chloroethyl acetate (0.08 mL, 1.0 eq, 0.73 mmol), K_2CO_3 (0.10 g, 1.0 eq, 0.73 mmol) and THF (10 mL). The solution was then left to reflux for 96 h at 66 °C while stirring. The reaction mixture turned deep brown and was then quenched with saturated NH_4Cl (0.30 mL). The deep brown solution was then extracted with EtOAc (3 × 15 mL) and the organic layer dried using $MgSO_4$, filtered and concentrated *in vacuo*. The crude material was purified using column chromatography with EtOAc:Hexane gradient starting at 10% as eluent to obtain the product **27**, a yellow crystalline solid, in a yield of 51% (83 mg).

This reaction was then scaled up by a factor of five and was successful in producing compound **27**, in a yield of 48% (0.39 g).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.66 (d, *J* = 8.3 Hz, 1H, Ar), 6.27 (d, *J* = 2.3 Hz, 1H, Ar), 6.05 (dd, *J* = 8.3, 2.3 Hz, 1H, Ar), 5.86 (s, 2H, -OCH_2O-), 4.24 (q, *J* = 7.1 Hz, 2H, O-$\underline{CH_2}CH_3$), 3.84 (s, 2H, -$NH\underline{CH_2}$-), 1.29 (t, 7.1 Hz, -CH_3).^{13}C NMR (100 MHz, $CDCL_3$) δ 171.06, 148.38, 142.53, 140.33, 108.58, 104.71, 100.67, 96.42, 61.28, 46.85, 14.15. HRMS: calculated mass for $C_{11}H_{14}NO_4$ [M+H] 224.0923; found 224.0923 *m/z*. ATS FT-IR cm^{-1}: 781, 807, 843, 902, 929, 1021, 1037, 1194, 1239, 1487, 1729, 2847, 3385. MP: 40-45°C. Confirmed by literature.[51]

3.2.1.2 *Synthesis of Ethyl 3-(benzo[d][1,3]dioxol-5-ylamino) propanoate (28)*

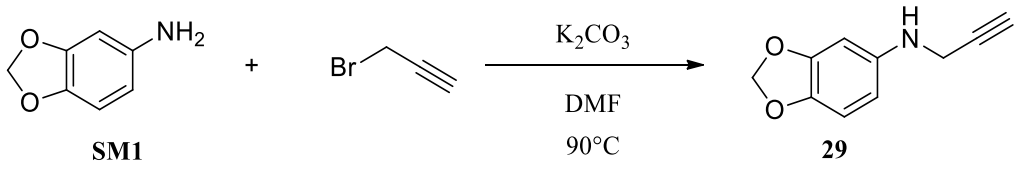


***Scheme 13**: Synthesis of ethyl ester functionalized aniline (**28**)*

3,4-(Methylenedioxy)aniline **SM1** (0.50 g, 1.0 eq, 3.7 mmol) and ethyl acrylate (0.40 mL, 1.0 eq, 3.7 mmol) were added to a 10 mL round bottom flask. This was then placed under conventional heating for 72 hours at 60 ˚C while stirring. A TLC plate of the final compound showed the formation of a distinct product peak. The resulting mixture was diluted with DCM and wet loaded onto a gravity column, where EtOAc: Hexane, started at 10%, was used as eluent. The final compound, **28**, a yellow-orange oil was obtained in a yield of 56% (0.48 g).

^{1}H NMR (600 MHz, $CDCl_3$) δ 6.62 (d, *J* = 8.3 Hz, 1H, Ar), 6.23 (d, *J* = 2.3 Hz, 1H, Ar), 6.04 (dd, *J* = 8.3, 2.3 Hz, 1H, Ar), 5.82 (s, 2H, -OCH_2O-), 4.13 (q, *J* = 7.1 Hz, 2H, O$\underline{CH_2}CH_3$), 3.34 (t, *J* = 6.3 Hz, 2H, NH$\underline{CH_2}CH_2$), 2.56 (t, *J* = 6.4 Hz, 2H, $NHCH_2\underline{CH_2}$), 1.23 (t, *J* = 7.2 Hz, 3H, $OCH_2\underline{CH_3}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.32, 148.35, 143.07, 139.99, 108.58, 105.01, 100.57, 96.46, 60.58, 40.63, 33.75, 14.14. HRMS: calculated mass for $C_{12}H_{16}NO_4$ [M+H] 238.1079; found 238.1071 *m/z*. ATS FT-IR cm^{-1}: Product showed signs of degradation, potentially due to storage conditions, making IR inconclusive. Further methods used for synthesis are discussed in Chapter 2 in **Table 2**, page 38.

3.2.1.3 *Synthesis of N-(but-3-yn-1-yl) benzo[d][1,3]dioxol-5-amine (29)*



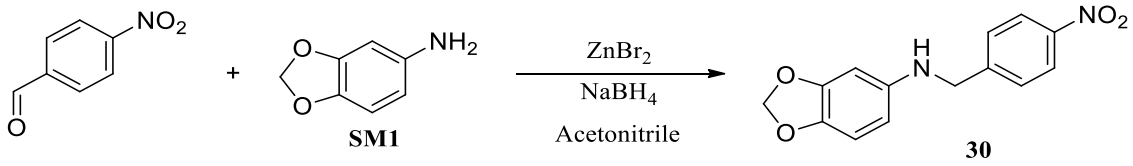
***Scheme 14**: Synthesis of propyne functionalized aniline (**29**)*

3,4-(Methylenedioxy)aniline **SM1** (0.10 g, 1.0 eq, 0.73 mmol) was added to propargyl bromide (0.11 mL, 1.0 eq, 0.73 mmol), K_2CO_3 (0.10 g, 1.0 eq, 0.73 mmol) and DMF (5.0 mL). The solution was

then left to reflux for 2 hours at 90 °C while stirring. The reaction mixture turned deep brown and was then quenched with saturated NH_4Cl (0.5 mL) and water (5.0 mL) and concentrated *in vacuo*. The deep brown solution was then extracted with EtOAc (3 × 15 mL) and the organic layer dried using $MgSO_4$, filtered and concentrated *in vacuo*. The crude material was purified using column chromatography with EtOAc:Hexane gradient starting at 10% as eluent to obtain the product **29**, a yellow oil, in a yield of 72% (92 mg). This reaction was repeated and scaled up by a factor of five with similar results (71% yield, 0.91 g).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.69 (d, *J* = 8.3 Hz, 1H, Ar), 6.32 (d, *J* = 2.3 Hz, 1H, Ar), 6.13 (dd, *J* = 8.3, 2.3 Hz, 1H, Ar), 5.87 (s, 2H, -OCH_2O-), 3.87 (d, *J* = 2.4 Hz, 2H,-$NHCH_2C$-), 3.62 (s, 1H, -NH-), 2.22 (s, 1H, -CCH).^{13}C NMR (100 MHz, $CDCl_3$) δ 148.35, 142.42, 140.57, 108.56, 105.45, 100.73, 96.86, 81.09, 71.38, 34.59. HRMS: calculated mass for $C_{10}H_{10}NO_2$ [M+H] 176.0712, found- 176.0706 *m/z*. ATS FT-IR cm-1: 660, 708, 749, 970, 812, 836, 896, 917, 1031, 1068, 1111, 1119, 1149, 1199, 1229, 1258, 1314, 1371, 1438, 1455, 1482, 1502, 1632, 25891, 3253, 3390.MP: 76-78 °C.

3.2.1.4 *Synthesis of N-(4-nitrobenzyl) benzo[d][1,3]dioxol-5-amine (30)*

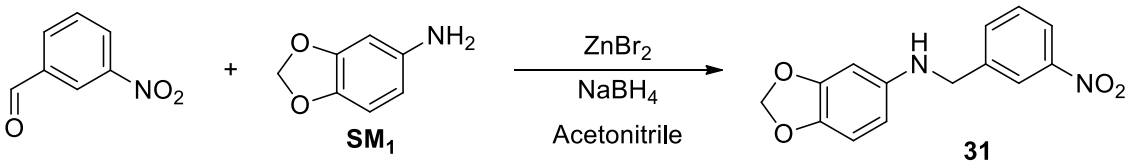


***Scheme 15**: synthesis of para-nitrobenzene functionalized aniline (**30**)*

4-Nitrobenzaldehyde (0.15 g, 1.0 eq, 1.0 mmol) and $ZnBr_2$ (0.25 g, 1,1 eq, 1.1 mmol) were added to acetonitrile and stirred for 20 minutes at room temperature. 3,4-(Methylenedioxyaniline) **SM1** (0.14 g, 1.0 eq, 1.0 mmol) was then added to the reaction mixture. This was stirred for 24 hours at room temperature. Sodium borohydride (76 mg, 2.0 eq, 2.0 mmol) was then added to the reaction mixture. The reaction was then left to stir at room temperature for 12 hours. The reaction was quenched with saturated NH_4Cl (0.50 mL) and water (5.0 mL) and concentrated *in vacuo*. The deep brown solution was extracted with EtOAc (3 × 15 mL) and the organic layer dried using $MgSO_4$, filtered and concentrated *in vacuo*. The crude material was purified using column chromatography with EtOAc:Hexane gradient starting at 10% as eluent to obtain the product **30**, a red crystalline material, in a yield of 60% (0.16 g).

1H NMR (300 MHz, $CDCl_3$) δ 8.28 – 8.05 (dd, *J* = 1.9 Hz, 2H, Ar-NO_2), 7.60 – 7.42 (dd, *J* = 8.3, 0.6, 2H, Ar-NO_2), 6.62 (d, *J* = 8.3 Hz, 1H, Ar), 6.21 (d, *J* = 2.4 Hz, 1H, Ar), 5.99 (dd, *J* = 8.3, 2.4 Hz, 1H, Ar), 5.83 (d, *J* = 0.5 Hz, 2H, -OCH_2O-), 4.40 (s, 2H, -$NHCH_2$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 148.52, 147.63, 147.21, 143.10, 140.17, 127.82, 123.93, 108.75, 104.55, 100.79, 96.13, 48.51. HRMS: calculated mass for $C_{12}H_{15}NO_4$ [M+H] 273.0875, found- 273.0868 *m/z*. ATS FT-IR cm^{-1}:741, 787, 826, 840, 1106, 1148, 1202,1234, 1291, 1342, 1396, 1428, 1463, 1502, 1515, 1605, 1630, 2846, 2903, 3076, 3388. MP: 58-60 °C.

3.2.1.5 *Synthesis of N-(3-nitrobenzyl)benzo[d][1,3]dioxol-5-amine (31)*



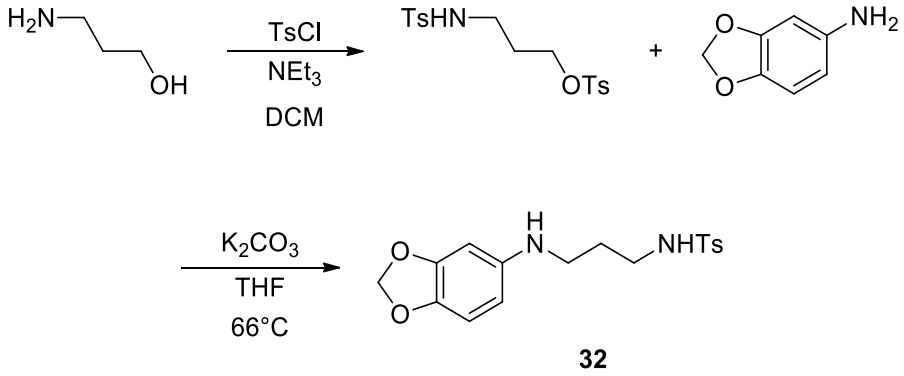
***Scheme 16**: Synthesis of meta-nitro benzene functionalized aniline (**31**)*

3-Nitrobenzaldehyde (0.15 g, 1.0 eq, 1.0 mmol) and $ZnBr_2$ (0.25 g, 1,1 eq, 1.1 mmol) were added to acetonitrile and stirred for 20 minutes at room temperature. 3,4-(Methylenedioxy)aniline **SM1** (0.14 g, 1.0 eq, 1.0 mmol) was then added to the reaction mixture. This was stirred for 24 hours at room temperature. Sodium borohydride (76 mg, 2.0 eq, 2.0 mmol) was then added to the reaction mixture. The reaction was left to stir at room temperature for 12 hours. The reaction was quenched with saturated NH_4Cl (0.50 mL) and water (5.0 mL) and concentrated *in vacuo*. The deep brown solution was extracted with EtOAc (3 × 15 mL) and the organic layer dried using $MgSO_4$, filtered and concentrated *in vacuo*. The crude material was purified using column chromatography with EtOAc:Hexane gradient starting at 10% as eluent to obtain the product **31**, a red crystalline material, in a yield of 70% (0.19 g).

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.21 (dd, *J* = 2.0 Hz, 1H, $ArNO_2$), 8.09 (dd, *J* = 8.2, 2.4 Hz, 1H, $ArNO_2$), 7.69 (d, *J* = 7.6 Hz, 1H, $ArNO_2$), 7.49 (dd, *J* = 8.7, 7.1 Hz, 1H, $ArNO_2$), 6.63 (dd, *J* = 8.3, 1.6 Hz, 1H, Ar), 6.22 (dd, *J* = 2.0 Hz, 1H, Ar), 6.01 (dd, *J* = 8.3, 2.0 Hz, 1H, Ar), 5.83 (s, 2H, -OCH_2O-), 4.38 (s, 2H, -$NHCH_2$), 4.05 (s, 1H, -NH). HRMS: calculated mass for $C_{14}H_{13}N_2O_4$ [M+H] 273.0875, found-

278.0871 *m/z*. ATS FT-IR cm^{-1}:787, 840, 1033, 1193, 1343,1484, 1500, 1515, 1604, 1629, 2846, 2904, 3387. MP: 52-56 °C.

3.2.1.6 *Synthesis of N-(3-(benzo[d][1,3]dioxol-5-ylamino)propyl)-4-methylbenzenesulfonamide (32)*



***Scheme 17**: Synthesis of tosyl protected propyl amine functionalized aniline (**32**)*

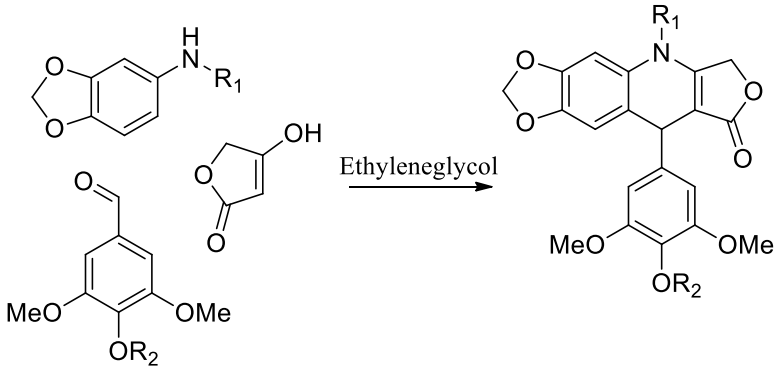
Tosyl chloride (1.0 g, 2.0 eq, 5.3 mmol) and 1-aminopropanol (0.20 g, 0.20 mL, 1.0 eq, 2.6 mmol) were added to DCM (10 mL) and stirred for four hours at room temperature. The reaction was then quenched by dissolution. The product was then loaded onto silica gel and purified using column chromatography using an EtOAc:Hexane gradient starting at 20% EtOAc was used as eluent to obtain the product, a white crystalline solid, in a yield of 22% (0.22 g). HRMS: calculated mass for $C_{17}H_{22}NO_5S_2$ [M+H] 384.0939; found 384.0932 *m/z* and confirmed by comparison to literature.[54]

This product, 3-(4-methylphenylsulfonamido)propyl 4-methylbenzenesulfonate (0.22 g. 1 eq, 0.6 mmol) was then added to 3,4-(methylenedioxy)aniline **SM1** (78 mg, 1.0 eq, 0.6 mmol) and K_2CO_3 (9.2 g, 1.2 eq, 0.7 mmol) in THF and stirred for 12 hours at 60 °C. The reaction mixture turned deep brown and was then quenched with saturated NH_4Cl (0.30 mL). The solution was extracted with EtOAc (3 × 15 mL) and the organic layer dried using $MgSO_4$, filtered and concentrated *in vacuo*. The crude material was purified using column chromatography with EtOAc:Hexane gradient starting at 10% used as eluent to obtain the product **32**, which co-eluted with starting material, in a total yield of 8% (16 mg) this reaction was repeated and was a yield of 50 mg crude product, which was used in the subsequent reaction.

^{1}H NMR (300 MHz, $CDCl_3$) δ 6.61 (d, *J* = 8.2 Hz, 3H), 6.28 (d, *J* = 2.3 Hz, 3H), 6.12 (dd, *J* = 8.2, 2.3 Hz, 3H), 5.85 (s, 6H), 4.12 (q, *J* = 7.1 Hz, 1H), 3.28 – 3.16 (m, 1H), 3.24 (s, 2H), 2.44 (d, *J* = 9.3 Hz, 1H), 1.26 (t, *J* = 14.3 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 148.73, 141.93, 140.89, 130.29, 127.58, 109.10, 107.41, 101.19, 98.61, 78.01, 77.58, 77.16, 60.94, 47.51, 21.58, 14.74, 1.56. ATS FT-IR cm^{-1}: 662, 706, 776, 811, 858, 900, 929, 1020, 1050, 1063, 1094, 1162, 1290, 1302, 1322, 1381, 1444, 1462, 1477, 2867, 2949, 2126, 3498. Mass spectral data was not obtained due to degradation of the product at the time of analysis, as a result, the product remains unidentified.

3.2.2 **4-aza-podophyllotoxin analogues**

Scheme 18 shows the generalized scheme used for the synthesis of the 4-aza-podophyllotoxins in Library 1. All reactions were performed in the presence of ethyleneglycol and 4-chloroaniline, unless otherwise stated.



Scheme 18: *Generalized method for the synthesis of 4-aza-podophylltoxin analogues; generalized multicomponent reaction*

The synthesis of the 4-aza-podophyllotoxins are represented in **Tables 7** and **8**. All synthesis followed the general procedure described below, unless stated otherwise.

The 4-aza-podophyllotoxin analogues below were synthesized in Library 1 from their respective anilines, **29-32.** These reactions were performed under air atmosphere, unless otherwise specified.

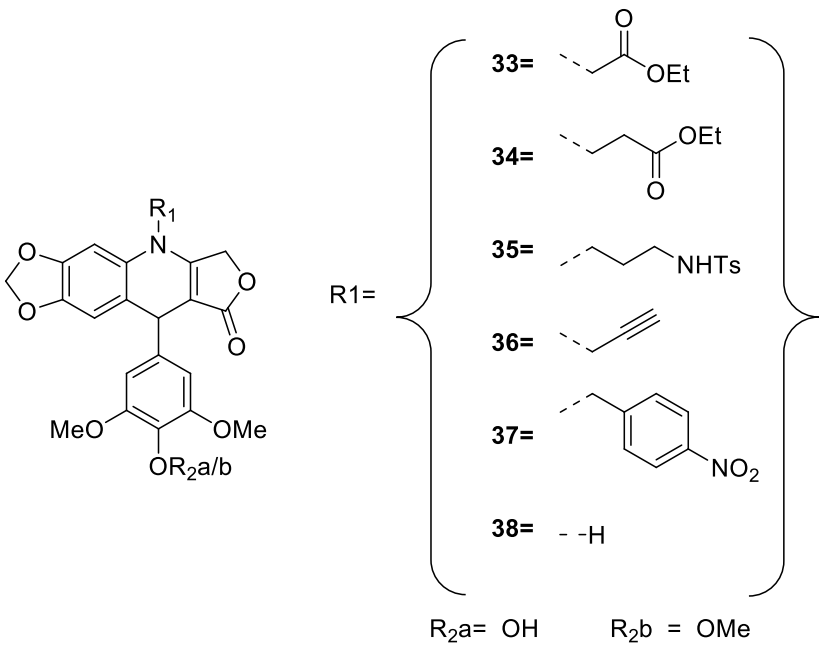


Figure 20: *4-aza-podophyllotoxin analogues synthesized (**33-38**)*

Synthetic procedure for compounds A

The functionalized aniline **(27-32)** (1.0 eq, 1.0 mmol), syringaldehyde (0.18 g, 1.0 eq, 1.0 mmol), tetronic acid (0.11 g, 1.0 eq, 1.0 mmol), 4-chloroaniline (13 mg, 0.1 eq, 0.1 mmol) and ethyleneglycol (1.5 mL) were added to a 10 mL microwave reaction vessel. The solution was heated to 196 °C for 30 seconds while stirring to dissolve reactants. Following this, the reaction vessel was placed under microwave irradiation and heated to the temperatures and time periods designated in **Table 7.** The products (**33a-39a**), were recovered via filtration and washed with 3 × 5 mL cold ethanol. The precipitate was then recrystallized in ethanol and recovered by filtration and washed again with cold ethanol (3 × 5 mL). Compound characterization for individual products follows below.

Table 8: *Methods used for the synthesis of compounds **33a-38a***

Product	Time in minutes	Temperature in °C	Heating method	Yield in %
33a	120	180	MWI	44
34a	120	180	MWI	8
35a	120	75	MWI	36
36a	7.5	110	MWI	32
37a	0.5	198	Conventional	61
38a	10	150	MWI	43

3.2.2.1 *Analysis of ethyl 2-[9-(4-hydroxy-3,5-dimethoxyphenyl)-8-oxo-6,9-dihydro-[1,3]dioxolo[4,5-g] furo[3,4-b] quinolin-5(8H)-yl]acetate (33a)*

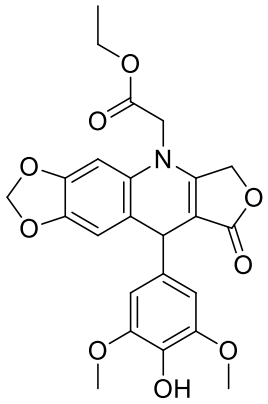


Figure 21**: Structure of methyl ester functionalised compound **33a

The best yield obtained was 44% (0.21 g).

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.13 (s, 1H, -OH, E ring), 6.82 – 6.70 (m, 2H, Ar, B-Ring), 6.53 (s, 2H, -OCH_2O-), 5.95 (dd, *J* = 16.2, 1.0 Hz, 2H, Ar, E ring), 5.06 (d, *J* = 16.0 Hz, 1H, -CCH_2O-), 4.90 (dd, *J* = 16.0, 1.1 Hz, 1H, -CCH_2O), 4.78 (s, 1H, CCHC, C ring), 4.72 (d, *J*=18.6 Hz, 1H, NCH_2C), 4.56 (d, *J* = 18.6 Hz, 1H, NCH_2C), 4.18 (q, *J* = 7.1 Hz, 2H, -CH_2CH_3), 3.70 (s, 6H, 2×-OCH_3, E ring), 1.23 (t, *J* = 7.1 Hz, 3H, CH_2CH_3). ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.3, 168.6, 159.49, 147.9, 146.8, 143.4, 137.2, 134.3, 131.4, 119.4, 109.9, 105.1, 102.3, 96.9, 95.5, 65.23, 61.3, 56.0, 47.5, 14.0. ATS FT-IR cm^{-1}: 932, 986, 1016, 1054, 1115, 1205, 1235, 1434, 1452, 1462, 1471, 1514, 1615, 1660, 1744, 1731, 3511. HRMS: calculated mass for $C_{24}H_{24}NO_9$ [M+H] 470.1451; found 470.1446 *m/z*. MP 235-237 °C.

3.2.2.2 *Analysis of ethyl 3-(9-(4-hydroxy-3,5-dimethoxyphenyl)-8-oxo-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-5(6H,8H,9H)-yl)propanoate (34a)*

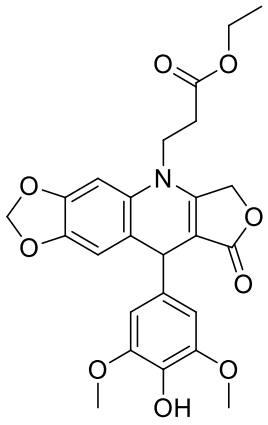


Figure 22**: Structure of ethyl ester functionalized compound **34a

The best yield obtained was 8% (39 mg). This reaction was repeated with similar results.

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.77 (s, 1H, OH, E ring), 7.52 (s, 1H, Ar, B ring), 7.13 (s, 1H, Ar, B ring), 6.69 (s, 2H, Ar, E ring), 6.75 – 6.54 (m, 1H, Ar, E ring), 6.29 (s, 2H, Ar, A ring), 5.41 (s, 2H-CCH_2O-), 3.75 (s, 3H, 2 × OCH_3, E ring), 3.88 – 3.64 (m, 3H, $CH_2\underline{CH_3}$), 3.43 (s, 2H, CH_2CH_2), 1.28 – 1.13 (t, 2H, -$\underline{CH_2}CH_3$). ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.44, 163.48, 153.45, 150.48, 149.01, 148.93, 148.15, 136.82, 124.08, 122.33, 112.43, 108.03, 105.41, 103.49, 102.37, 69.27, 63.25, 56.63. ATS FT-IR cm^{-1}: 858, 1033, 1113,1190, 1267,1332, 1368, 1421, 1451, 1503, 1555, 1608, 1655, 1722, 2940, 3496. HRMS: calculated mass for $C_{25}H_{25}NO_9$ [M+H] 484.16; found 484.1596 *m/z*. ATS FT-IR cm^{-1}: 858, 1033, 1113,1190, 1267,1332, 1368, 1421, 1451, 1503, 1555, 1608, 1655, 1722, 2940, 3496. MP 240-242 °C.

3.2.2.3 *Analysis for unknown compound initially thought to be 35a*

An unknown mixture of two products was obtained when the synthesis of **35a** was attempted. The individual products could not be separated, despite further attempts at recrystallization. The unknown minor product appeared in the ^{1}H NMR spectra in very small amounts, making integration inaccurate for these peaks. The peaks have been designated ‘m’ for the minor product and ‘M’ for the major product in the ^{1}H NMR spectrum. Initial synthesis of compound **35a**, was not successful, this is discussed in further detail in

Chapter 2. Optimization for the synthesis for *N*-functionalized aniline **32** will need to be performed before further attempts at synthesis can be undertaken. The analytical data for this mixture is presented below.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, m), 8.19 (s, 2H, M), 7.84 (s, 1H,), 7.21 (s, m), 6.54 (s, m), 4.67 (s, 1H, M), 3.86 (s, 2H, M), 3.39 (s, m), 2.08 (s, m), 1.31 – 1.02 (m, m). Potentially corresponding to major peak at 398.2332 *m/z* in the mass spectrum. ^{13}C NMR (101 MHz, DMSO-d_6) δ 196.31, 195.00, 190.93, 180.42, 176.70, 170.68, 168.47, 154.11, 151.90, 147.90, 147.43, 144.63, 141.87, 135.68, 133.42, 126.93, 123.78, 122.48, 113.83, 113.03, 106.86, 104.44, 98.05, 72.32, 71.30, 67.41, 62.58, 57.01, 56.01, 55.95, 55.86, 55.81, 54.82, 32.20, 30.50. HRMS: calcd for $C_{30}H_{30}N_2O_9S$ mass [M+H] 595.1750 *m/z* found minor peak at 595.0836 *m/z*, and major peaks at 546.0695, and 398.2332 *m/z*. ATS FT-IR cm^{-1}:686, 705, 877, 983, 1020,1053,1108, 1138, 1161, 1228, 1275, 1324, 1367, 1442, 1493, 1558, 1667, 1745, 3229. MP: 186-192 °C.

3.2.2.4 *Analysis of 9-(4-hydroxy-3,5-dimethoxyphenyl)-5-(prop-2-yn-1-yl)-6,9-dihydro-[1,3]dioxolo [4,5-g]furo[3,4-b]quinolin-8(5H)-one (36a)*

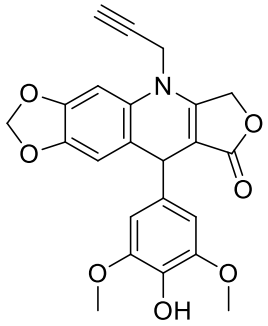


Figure 23: *Structure of propargyl functionalized compound* ***36a***

The best yield obtained was 32% (0.14 g). This reaction was repeated and scaled up by a factor of 3 with similar results obtaining a yield of 32% (0.38 g), for use in click reactions.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.16 (s, 1H, -OH, E ring), 6.98 (s, 1H, Ar, A ring), 6.77 (s, 1H, Ar A ring), 6.42 (d, *J* = 1.7 Hz, 2H, -OCH$_2$O-), 6.00 (s, 1H, Ar, E ring), 5.95 (s, 1H, Ar, E ring), 5.19 (d, *J* = 16.1 Hz, 1H, -CCH$_2$O-), 4.96 (d, *J* = 16.0 Hz, 1H, -CCH$_2$O-), 4.78 (s, 1H, -CCHC-, C ring), 4.61 (dd, *J* = 6.3, 2.5 Hz, 2H, -NCH$_2$C), 3.67 (s, *J* = 1.6 Hz, 6H, 2×-OCH$_3$, E ring), 3.40 (d, *J* = 2.2 Hz, 1H, -CCH).

^{13}C NMR (75 MHz, DMSO-d_6) δ 216.46, 172.29, 159.36, 148.36, 147.18, 144.00, 137.39, 134.79, 131.05, 120.22, 110.29, 105.26, 101.91, 97.92, 96.97, 78.76, 76.14, 65.58, 63.26, 56.47, 36.09. HRMS: calculated mass for $C_{23}H_{20}NO_7$ [M+H] 422.1240; found 422.1238 *m/z*. ATS FT-IR cm^{-1}:686, 707, 768, 794, 832, 873, 937, 978, 1015, 1044, 1111, 1204, 1238, 1318, 1427, 1454, 1478, 1608, 1643, 1726, 2898, 3245, 3554. MP 210-212 °C.

3.2.2.5 *Analysis of 9-(4-hydroxy-3,5-dimethoxyphenyl)-5-(4-nitrobenzyl)-6,9-dihydro [1,3]dioxolo [4,5-g]furo[3,4-b]quinolin-8(5H)-one (37a)*

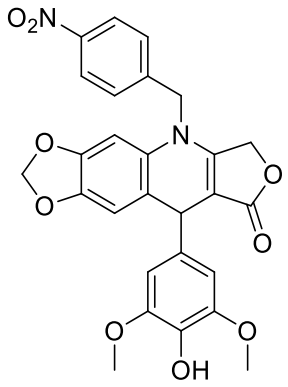


Figure 24**: Structure of nitrobenzene functionalized compound **37a

The best yield obtained was 61% (0.32 g). This was achieved under conventional heating for 30 seconds.

^{1}H NMR (300 MHz, DMSO-d_6) δ 8.27 – 8.14 (m, 2H, Ar-NO_2), 7.67 – 7.54 (m, 2H, Ar-NO_2), 6.76 (d, J = 0.6 Hz, 1H, Ar, B ring), 6.67 (s, 1H, Ar, B ring), 6.47 (s, 2H, -OCH_2O-), 5.90 (s, 2H, 2 × Ar, E ring), 5.26 – 4.85 (m, 5H, -CCHC-,-CCH_2O-, -NCH_2C-), 3.71 (s, 6H, -OCH_3). ^{13}C NMR (75 MHz, DMSO-d_6) 206.22, 171.76, 159.21, 147.65, 146.67, 146.41, 144.36, 143.21, 136.75, 134.08, 130.56, 127.42, 123.62, 119.23, 109.82, 104.78, 101.14, 96.08, 96.04, 65.14, 62.51, 55.71, 48.20, 40.08, 39.80, 39.68, 39.52, 39.24, 38.96, 38.68, 38.41, 30.41. HRMS: calculated mass for $C_{27}H_{23}N_2O_9$ [M+H] 519.1404; found 519.1406 *m/z*. ATS FT-IR cm^{-1}: 728, 765, 872, 929, 980, 994, 1032, 1111, 1196, 1229, 1320, 1342, 1459, 1476, 1515, 1612, 1655, 1729, 2876, 2935, 3341, 3482. MP 196-200 °C.

3.2.2.6 *Analysis of 9-(4-hydroxy-3,5-dimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one (38a)*

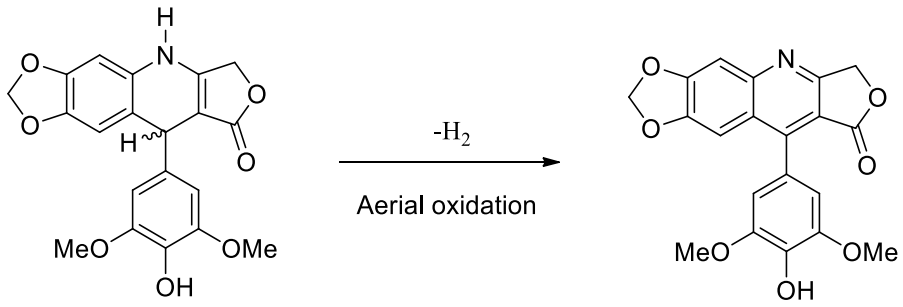


***Figure 25**: Unfunctionalized compound **38a** and oxidized form of the compound*

The best yield obtained was 43% (0.16 g).

NMR reported for the mixture of both forms of 38a: ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.83 – 9.77 (s, 2H), 8.82 – 8.76 (s, 1H), 8.19 – 8.13 (s, 2H), 7.54 – 7.46 (s, 1H), 7.17 – 7.11 (s, 1H), 6.74 – 6.68 (s, 1H), 6.67 – 6.61 (s, 2H), 6.55 – 6.41 (d, J = 21.8 Hz, 6H), 6.31 – 6.25 (s, 1H), 5.99 – 5.87 (dd, J = 14.1, 1.0 Hz, 4H), 5.43 – 5.37 (s, 1H), 5.03 – 4.92 (d, J = 15.6 Hz, 2H), 4.89 – 4.78 (d, J = 15.7 Hz, 2H), 4.82 – 4.74 (s, 2H), 3.79 – 3.73 (s, 3H), 3.73 – 3.66 (s, 12H), 3.43 – 3.37 (s, 1H).^{13}C NMR (75 MHz, DMSO-d_6) δ 172.63, 163.45, 158.68, 153.42, 148.34, 148.15, 146.79, 143.62, 137.89, 136.83, 134.75, 130.70, 122.34, 117.50, 112.39, 110.02, 108.04, 105.65, 101.58, 97.68, 94.84, 65.33, 56.63, 56.50. HRMS: calculated mass for $C_{20}H_{16}NO_7$ [M+H] 382.0927; found 382.0922 *m/z*. ATS FT-IR cm^{-1}:684, 758, 778, 790, 807, 818, 851, 866, 933, 1015, 1032, 1066, 1103, 1115, 1165, 1192, 1246, 1303, 1329, 1348, 1425, 1460, 1481, 1498, 1551, 1621, 1656, 1731, 3334. MP 234-236 °C. ^{13}C spectrum of unoxidized product was not obtained.

Synthetic procedure for compounds B

The functionalized aniline **(27-32)** (1.0 eq, 1.0 mmol), trimethoxy benzaldehyde (0.20 g, 1.0 eq, 1.0 mmol), tetronic acid (0.11 g, 1.0 eq, 1.0 mmol), 4-chloroaniline (13 mg, 0.10 eq, 0.10 mmol) and ethyleneglycol (1.5 mL) were added to a 10 mL microwave reaction vessel. The solution was then heated to 196 °C for 30 seconds while stirring to dissolve reactants. Following this the reaction vessel was placed under microwave irradiation and heated to the temperature, and time period designated in **Table 8**. The product **(33b-39b)**, was recovered via filtration and washed with 3 × 5 mL cold ethanol. The precipitate

was then recrystallized in ethanol and recovered by filtration and washed with cold ethanol (3 × 5 mL). Compound characterization for individual products follows below.

Table 9: *Methods used for the synthesis of* ***33b-38b***

Product	Time in minutes	Temperature in °C	Heating method	Yield in %
33b	120	180	MWI	35
34b	120	180	MWI	14
35b	35	75	MWI	87
36b	10	150	MWI	66
37b	0.5	198	Conventional	15
38b	10	150	MWI	28

3.2.2.7 *Analysis of Ethyl 2-[8-oxo-9-(3,4,5-trimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5g]furo[3,4-b]quinolin-5(8H)-yl]acetate (33b)*

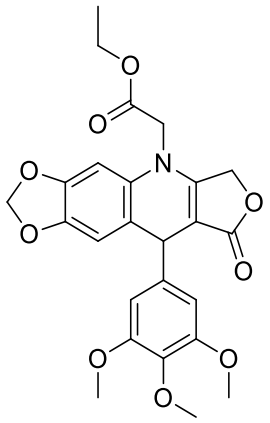


Figure 26*: Structure of methyl ester functionalized compound* ***33b***

The best yield obtained was 35% (0.17 g).

^{1}H NMR (400 MHz, DMSO-d_6) δ 6.79 (s, 1H, Ar, B ring), 6.75 (d, J = 0.6 Hz, 1H, Ar, B ring), 6.59 (s, 2H, -OCH$_2$O-), 5.98 (d, J = 1.0 Hz, 1H, Ar, E ring), 5.93 (d, J = 1.1 Hz, 1H, Ar, E ring), 5.14 – 4.55 (m, 5H, CCHC-,-CCH$_2$O-, -NCH$_2$C-),4.18 (q, J = 7.1 Hz, 2H, O<u>CH$_2$</u>CH$_3$), 3.72 (s, 6H, 2 ×-OCH$_3$, E ring), 3.59 (s, 3H, -OCH$_3$), 1.22 (t, J = 7.1 Hz, 3H,-CH$_2$<u>CH$_3$</u>). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.64, 162.69, 152.89, 152.42, 149.82, 147.56, 137.56, 127.43, 123.22, 111.79, 106.97, 104.73, 102.88, 101.52, 68.71, 62.57, 59.91, 55.84, 55.53, 39.99, 39.94, 39.78, 39.73, 39.57, 39.52, 39.31, 39.10, 38.90, 38.69.ATS FT-IR cm^{-1}: 932, 986, 1016, 1054, 1115, 1205, 1235, 1434, 1452, 1462, 1471, 1514, 1615, 1660, 1744,

1731, 3511. HRMS: calculated mass for $C_{25}H_{26}NO_9$ [M+H] 484.1608; found 484.1602 *m/z*. MP: 224-228 °C.

3.2.2.8 *Analysis of ethyl 3-(8-oxo-9-(3,4,5-trimethoxyphenyl)-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-5(6H,8H,9H)-yl)propanoate (34b)*

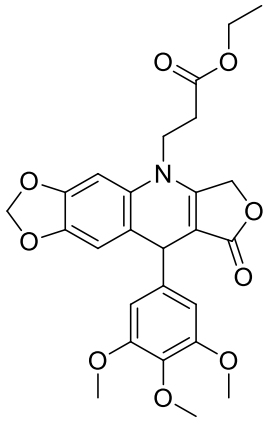


Figure 27**: Structure of ethyl ester functionalized compound **34b

The best yield obtained was 14% (69 mg)

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.55 (s, 1H, Ar, B ring), 7.08 (s, 1H, Ar, B ring), 6.74 (s, 2H, -OCH_2O-), 6.30 (s, 2H, Ar, E ring), 5.44 (s, 2H, -CCH_2O-), 3.87 – 3.57 (m, 16H, 3 × -OCH_3, N$\underline{CH_2}CH_2$, O$\underline{CH_2}CH_2$, $OCH_2\underline{CH_3}$), 3.39 (s, 3H, -OCH_3), 1.21 (m, 2H, $NCH_2\underline{CH_2}$).^{13}C NMR (100 MHz, DMSO-d_6) δ 168.30, 163.35, 153.56, 153.09, 150.48, 148.22, 138.22, 128.09, 123.88, 112.45, 107.63, 105.39, 103.54, 102.18, 69.37, 63.23, 60.57, 56.51, 56.19. HRMS: calculated mass for $C_{26}H_{26}NO_9$ [M+H] 496.1608 found 496.1609 *m/z*. ATS FT-IR cm^{-1}: 857, 932, 1004, 1120, 1151, 1187, 1241, 1254, 1297, 1322, 1365, 1414, 1457, 1498, 1557, 1598, 1611, 1726, 2839, 2931, 3001, 3380, 3417, 3497. MP 236-239 °C.

3.2.2.9 *Analysis of unknown compound (35b)*

Initial synthesis of compound **35b**, was not successful, this is discussed in further detail in **Chapter 2**. Optimization for the synthesis for *N*-functionalized aniline **32** will need to be performed before further attempts at synthesis can be undertaken. The analytical data for this mixture is presented below.

^{1}H NMR (300 MHz, DMSO-d_6) δ 6.48 (d, *J* = 3.3 Hz, 2H), 6.34 (s, 1H), 6.18 (s, 1H), 5.87 (dd, *J* = 5.0, 1.0 Hz, 3H), 5.30 (s, 1H), 4.66 – 4.48 (m, 2H), 3.92 – 3.72 (m, 3H), 3.69 (s, 1H), 3.70 – 3.55 (m, 6H), 3.05 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.26, 159.57, 153.28, 153.12, 147.33, 146.76, 144.08, 142.73, 140.60, 139.17, 138.09, 136.57, 136.54, 131.09, 120.83, 119.74, 110.27, 109.37, 107.19, 104.92, 101.98, 100.79, 97.68, 97.08, 95.70, 82.66, 78.73, 76.17, 73.81, 65.67, 63.25, 60.45, 60.33, 56.27, 44.97, 36.13, 33.86. HRMS: calculated mass for $C_{32}H_{33}N_2O_9S$ [M+H] 608.1906 major peaks observed at 595.2324, 551.1171 and 455.0823 *m/z*. ATS FT-IR cm^{-1}: 688, 705, 786, 794, 830, 940, 977, 1002, 1014, 1047, 1126, 1129, 1203, 1229, 1314, 1419, 1449, 1478, 1505, 1588, 1611, 1644, 1729, 2893, 3224, 3254. MP 188-192 °C.

3.2.2.10 *Analysis of 5-(prop-2-yn-1-yl)-9-(3,4,5-trimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one (36b)*

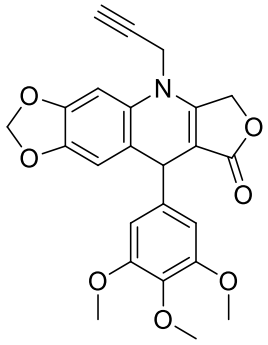


Figure 28**: Structure of propargyl functionalized compound **36b

The best yield obtained was 66% (0.29 g). This reaction was repeated and scaled up by a factor of 1.5 resulting in 50% yield (0.33g).

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.00 (s, 1H, Ar, B ring), 6.79 (s, 1H, Ar, B ring), 6.47 (s, 2H, -OCH_2O-), 6.02 (d, J = 1.0 Hz, 1H, Ar, E ring), 5.96 (d, J = 1.1 Hz, 1H, Ar E ring), 5.20 (d, *J* = 16.0 Hz, 1H,-CCH_2O), 4.97 (d, *J* = 16.0 Hz, 1H, -CCH_2O), 4.84 (s, 1H, CCHC, C ring), 4.70 – 4.53 (m, 2H, -NCH_2), 3.69 (s, 6H, 2 × -OCH_3, E ring), 3.59 (s, 3H, -OCH_3), 3.44 – 3.39 (m, 1H, -CCH). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.26, 159.33, 148.34, 147.15, 143.98, 137.36, 134.77, 131.02, 120.20, 110.27, 105.25, 101.89, 97.91, 96.95, 78.74, 76.12, 65.56, 63.24, 56.56, 56.44, 56.33, 36.07. HRMS: calculated mass for

$C_{24}H_{22}NO_7$ [M+H] 436.1396; found 436.1382 *m/z.* ATS FT-IR cm^{-1}: 688, 831, 940, 977, 997, 1013, 1047, 1125, 1203, 1229, 1315, 1420, 1436, 1454, 1477, 1589, 1610, 1643, 1728, 2836, 2892, 2944, 2970, 3224. MP 188-192 °C.

3.2.2.11 *5-(4-Nitrobenzyl)-9-(3,4,5-trimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one(37b)*

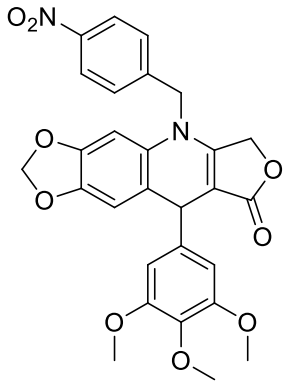


Figure 29**: Structure of nitrobenzene functionalized compound **37b

The best yield obtained was 15% (80 mg), achieved under conventional heating to 198 °C for 30 seconds.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.27 – 8.14 (m, 2H, Ar-NO_2), 7.65 – 7.55 (m, 2H, Ar-NO_2), 6.78 (d, *J* = 0.6 Hz, 1H, Ar, A ring), 6.68 (s, 1H, Ar, A ring), 6.52 (s, 2H, -OCH_2O-), 5.98 – 5.85 (m, 1H, Ar, E ring), 5.80 (s, 1H, -CCHC-), 5.19 (m, 2H, CCH_2O), 5.09 – 4.93 (m, 2H, -NCH_2C-), 3.73 (s, 6H, 2×OCH_3), 3.61 (s, 3H, -OCH_3).^{13}C NMR spectrum is not included as the majority of the product was sent for biological testing at YSU. Insufficient yield remained for accurate analysis. When running the sample for 24 hours, most peaks had not cleared the baseline. Recovery of the product from YSU was not possible to enable further analysis. HRMS: calculated mass for $C_{28}H_{25}N_2O_9$ [M+H] 533.1560; found 533.1594 *m/z.* ATS FT-IR cm^{-1}: 728, 765, 872, 929, 980, 994, 1032, 1111, 1196, 1229, 1320, 1342, 1459, 1476, 1515, 1612, 1655, 1729, 2876, 2935, 3341, 3482. MP: 194-198 °C.

3.2.2.12 *Analysis of 9-(3,4,5-trimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one (38b)*

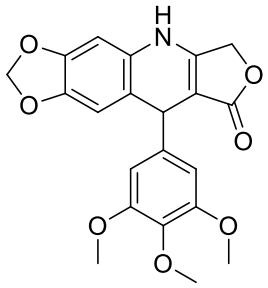
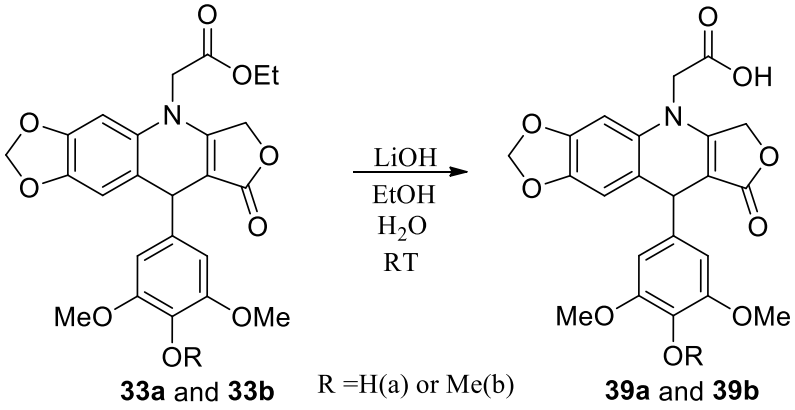


Figure 30**: Structure of unfunctionalized compound **38b

The best yield obtained was 28% (0.11 g).

^{1}H NMR (300 MHz, DMSO-d_6) δ 6.67 (d, J = 2.2 Hz, 1H, Ar, B ring), 6.53 (d, J = 2.2 Hz, 1H, Ar, B ring), 6.48 (d, J = 2.2 Hz, 2H, Ar, E ring), 5.93 (dd, J = 15.7, 2.2 Hz, 2H, -OCH_2O-), 4.98 (dd, J = 15.7, 2.2 Hz, 1H, -CCHC-), 4.90 – 4.78 (m, 2H, -CCH_2O-), 3.80 – 3.62 (m, 6H, 2 × -OCH_3), 3.60 (d, J = 2.2 Hz, 3H, -OCH_3).^{13}C NMR (100 MHz, DMSO-d_6) δ 172.16, 158.46, 152.83, 146.52, 143.28, 142.77, 130.37, 116.57, 109.58, 104.89, 101.21, 97.35, 94.19, 64.98, 59.91, 55.88. HRMS: calculated mass for $C_{21}H_{20}NO_7$ [M+H] 398.1240; found 398.1236 *m/z*. ATS FT-IR cm^{-1}: 684, 760, 837, 930, 1007, 1030, 1122, 1159, 1186, 1201,1235, 1325, 1418, 1461, 1478, 1501, 1555, 1591, 1612, 1644, 1721, 2939, 3080, 3222. MP: 210-214 °C. Consistent with literature.[37] ^{13}C spectrum of unoxidized product was not obtained.

3.2.3 Hydrolysis of 33a and 33b



Scheme 19: *Scheme to show the generalized synthesis of **39a** and **36b***

The synthesis of compounds **39a** and **39b** was attempted by reacting LiOH (0.012 g, 1.00 eq, 0.050 mmol), water (1.00 mL), compound **33a/33b** (1.00 eq, 0.05 mmol) and ethanol at room temperature for 12 hours. The reaction was then quenched using 1 M HCl (1.00 mL), and the organic layer extracted using 3 × 5 mL EtOAc. The product was concentrated *in vacuo*. Both **39a** and **39b** appeared to be orange solids. A sample was then run on a TLC plate and stained using Bromocresol green. Both products displayed clear yellow spots indicating the presence of **39b** and **39b,** which were obtained with yields of 99% (**39a**) and 43% (**39b**), respectively. The final product was obtained as a mixture. Analysis of the mixtures is presented below.

3.2.3.1 *Compound 2-(9-(4-hydroxy-3,5-dimethoxyphenyl)-8-oxo-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-5(6H,8H,9H)-yl)acetic acid (39a)*

^{1}H NMR (600 MHz, DMSO-d_6) δ 6.77 (s, 2H), 6.73 (s, 1H), 6.54 (s, 3H), 5.97 (d, J = 1.1 Hz, 2H), 5.92 (d, J = 1.0 Hz, 2H), 5.05 (d, J = 15.9 Hz, 2H), 4.90 (d, J = 16.0 Hz, 2H), 4.77 (s, 2H), 4.64 (s, 1H), 4.61 (s, 1H), 4.43 (s, 1H), 4.40 (s, 1H), 3.80 – 3.74 (m, 1H), 3.69 (s, 9H), 2.08 (s, 4H), 1.25 (s, 1H), 1.24 (s, 2H), 0.89 – 0.80 (m, 2H).^{13}C NMR (100 MHz, DMSO-d_6) δ 172.42, 170.40, 160.01, 148.26, 147.15, 143.75, 137.84, 134.57, 131.96, 119.86, 110.35, 105.38, 101.78, 96.94, 95.92, 65.71, 63.22, 56.37, 40.50, 40.36, 31.11, 22.51. HRMS: calculated mass for $C_{22}H_{20}NO_9$ [M+H] 442.1138; found 442.1134 *m/z*. ATR

FT-IR cm^{-1}: 789, 877, 987, 1032, 1113, 1208, 1360, 1478, 1507, 1612, 1649, 1715, 2922, 3371. MP :166-169 °C.

3.2.3.2 *Compound 2-(8-oxo-9-(3,4,5-trimethoxyphenyl)-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-5(6H,8H,9H)-yl)acetic acid (39b)*

^{1}H NMR (400 MHz, DMSO-d_6) δ 13.27 (s, 1H), 6.80 (d, *J* = 1.0 Hz, 1H), 6.75 (d, *J* = 0.7 Hz, 1H), 6.59 (d, *J* = 3.1 Hz, 2H), 5.98 (d, *J* = 1.0 Hz, 1H), 5.93 (d, *J* = 1.1 Hz, 1H), 5.06 (dd, *J* = 16.0, 5.0 Hz, 1H), 4.92 (d, *J* = 16.0 Hz, 1H), 4.86 – 4.77 (m, 1H), 4.64 (s, 1H), 4.34 (t, *J* = 5.1 Hz, 1H), 4.18 (q, *J* = 7.1 Hz, 1H), 3.71 (d, *J* = 4.5 Hz, 6H), 3.59 (d, *J* = 2.3 Hz, 3H), 1.22 (t, *J* = 7.1 Hz, 1H). The ^{13}C NMR spectrum is not presented because after 24 hours of data acquisition, the spectrum showed no peaks above the baseline for one of two reasons; either due to their being insufficient material for analysis or that the NMR spectroscopic instrument not being sufficiently sensitive. Alternatively, an issue with the data acquisition occurred – this spectrum will be re-run when material is resynthesized. This HRMS: calculated mass for $C_{23}H_{22}NO_9$ [M+H] 456.1295; found 456.1276 *m/z*. ATR FT-IR cm^{-1}: 931, 1031, 1120, 1197, 1239, 1361, 1418, 1463, 1503, 1584, 1651, 1738, 2853, 2922, 3405. MP:172-178 °C.

3.3 Click reactions used in the synthesis of Library 2

This section focuses on the synthesis of the azide alkyne cycloaddition products synthesized in Library 2. It begins with the synthetic procedures used to synthesize the desired azides, and their characterization. It then describes the methods used to perform the click reaction using products **36a** and **36b** from Library 1 as a starting point.

The azides represented in **Figure 27** were synthesized for use in the synthesis of Library 2.

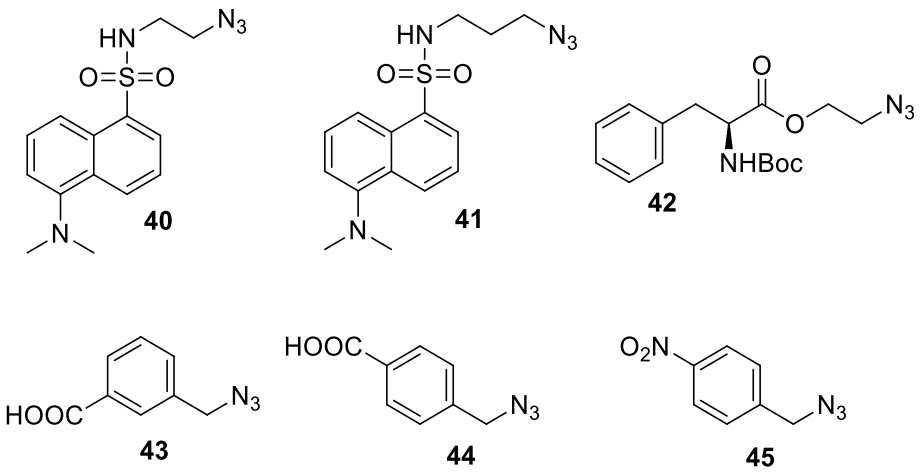
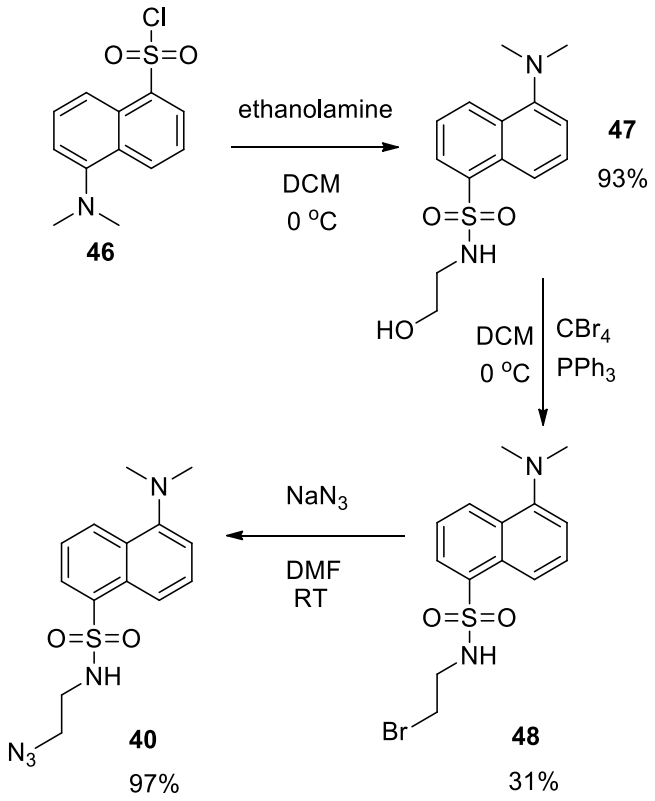


***Figure 31**: Azides synthesized for use in azide alkyl cycloaddition (**40-45**)*

3.3.1 Synthesis of azides 40-45

3.3.1.1 *Synthesis of N-(2-azidoethyl) naphthalene-1-sulfonamide (40)*



Scheme 20**: Synthesis of product **40** from dansyl chloride **46

Ethanolamine (0.21 g, 0.52 mL, 1.2 eq, 2.8 mmol) and triethylamine (0.37 g, 0.73 mL, 2.0 eq, 3.7 mmol) were added to DCM (5.0 mL) at 0 °C under N_2 gas and stirred for 5 minutes. Dansyl chloride, **46**, (0.50 g, 1.0 eq, 1.9 mmol) was dissolved in DCM (5 mL) and added dropwise over 15 minutes to the reaction mixture. This was stirred for 1.5 hours at 0 °C. The reaction was then quenched with saturated $NaCO_3$ (1.0 mL) and DCM (5.0 mL). Thereafter the organic layer was extracted with DCM (2 × 15 mL) and concentrated *in vacuo*. The yellow solution was dried using $MgSO_4$, filtered and concentrated *in vacuo*. The crude material was purified using column chromatography with EtOAc:Hexane gradient starting at 25% EtOAc was used as eluent to obtain the product (**47)**, a yellow crystalline solid in a yield of 93% (0.51g). HRMS: calculated mass for $C_{22}H_{19}NO_9$ [M+H] 295.1045; found 295.1114 *m/z*. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (m, 1H, Ar), 8.32 – 8.23 (m, 2H, Ar), 7.56 (m, 2H, Ar), 7.20 (dd, *J* = 7.5, 0.9 Hz, 1H, Ar),

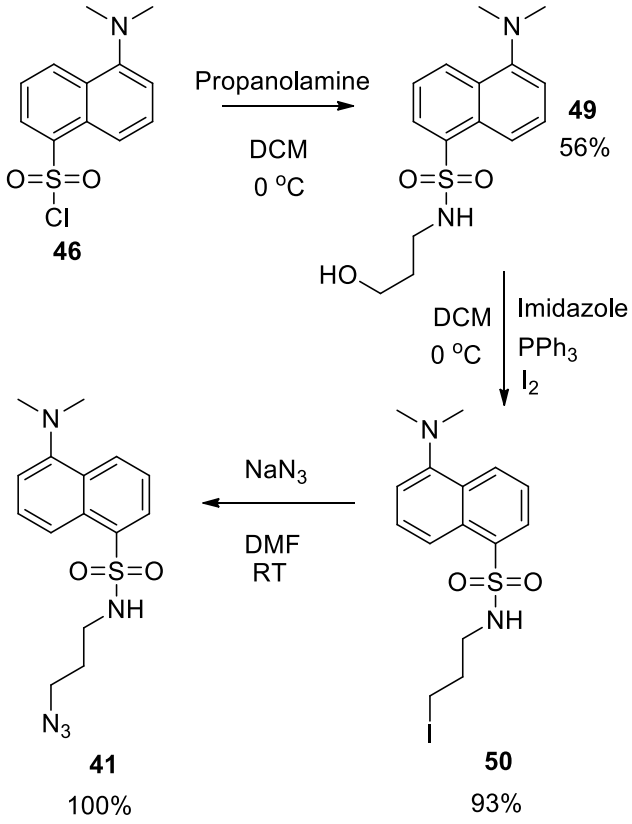
5.06 (m, *J* = 6.2 Hz, 1H, Ar), 3.61 (dd, *J* = 5.1 Hz, 2H, N$\underline{CH_2}CH_2$), 3.09 – 3.00 (m, 2H, CH_2CH_3), 2.90 (s, 6H, -$N(CH_3)_2$). ^{13}C NMR and IR spectroscopy have been omitted as the product was sufficiently characterized for further use.

Product **47** (75 mg, 1.2 eq, 0.25 mmol) and CBr_4 (92 mg, 1.3 eq, 0.28 mmol) was added to DCM (2.0 mL) at 0 °C under N_2. Triphenyl phosphine (74 mg, 1.3 eq, 0.28 mmol) in DCM (3 mL) was then added dropwise over 30 minutes. The reaction mixture was allowed to warm to room temperature while stirring for two hours. The crude mixture was concentrated *in vacuo*, hexane was added (10 mL), and the precipitate filtered off and washed with hexane. The elute was then concentrated *in vacuo* and purified using column chromatography with EtOAc:Hexane gradient starting at 25% EtOAc was used as eluent to obtain the product, a yellow oil, **48**, in a yield of 31% (29 mg). This reaction was repeated on a scale 5 times that of the initial experiment, yielding the product in 30% yield (0.14g)

Compound **48** (0.12 g, 1.0 eq, 0.30 mmol) was then reacted with sodium azide (20 mg, 1.0 eq, 0.30 mmol) in DMF for 5 hours starting at 0 °C and warming to room temperature under N_2. The reaction was quenched by dissolution by the addition of water. The product was dried in *vacuo* at low temperature. The product was then purified using flash chromatography with DCM: MeOH as elute. A pale-yellow oil, **40**, was obtained in a yield of 97% (31 mg).

1H NMR (400 MHz, $CDCl_3$) δ 8.58-8.55 (m, *J* = 8.5, 1.1 Hz, 1H, Ar), 8.32 – 8.23 (m, 2H, Ar), 7.56 (m, 2H, Ar), 7.20 (dd, *J* = 7.5, 0.9 Hz, 1H, Ar), 5.06 (t, *J* = 6.2 Hz, 1H, -NH), 3.61 (dd, *J* = 5.1 Hz, 2H,-N$\underline{CH_2}CH_2$-), 3.09 – 3.00 (m, 2H, -$NCH_2\underline{CH_2}$-), 2.90 (s, 6H, -$N(CH_3)_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.12, 134.46, 130.80, 129.96, 129.61, 129.48, 128.67, 123.14, 118.44, 115.37, 50.92, 45.39, 42.37. HRMS: calculated mass for $C_{14}H_{18}N_5O_2S$ [M+H] 320.1181; found 320.1173 *m/z*. ATS FT-IR cm^{-1}: 668, 697, 952, 1019, 1310, 1406, 1436, 1661, 2913, 2995, 3441. Liquid at room temperature (25 °C). Confirmed by comparison to literature.[64]

3.3.1.2 *Synthesis of N-(3-azidopropyl)-5-(dimethylamino)naphthalene-1-sulfonamide (41)*



Scheme 21: *Synthesis of product* ***41*** *from dansyl chloride* ***46***

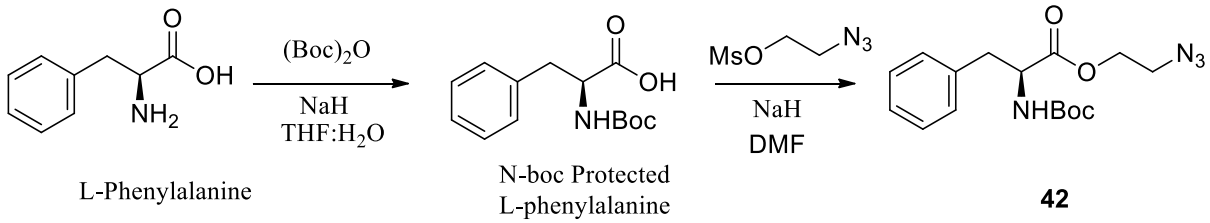
1-Amino propanol (0.42 g, 0.42 mL, 1.5 eq, 5.5 mmol) and triethylamine (0.37 g, 0.56 mL, 2.0 eq, 7.4 mmol) were added to DCM (5.0 mL) at 0 °C under N_2 gas and stirred for 5 minutes. Dansyl chloride, **46**, (1.0 g, 1.0 eq, 3.7 mmol) was then dissolved in DCM (10 mL) and added dropwise over 15 minutes to the reaction mixture. This was stirred for 1.5 hours at 0 °C. The reaction was then quenched with saturated $NaCO_3$ (1.0 mL) and DCM (5.0 mL). Thereafter the organic layer was extracted with DCM (2 × 15 mL) and concentrated *in vacuo*. The yellow solution was dried using $MgSO_4$, filtered and concentrated *in vacuo*. The crude material was purified using column chromatography with an EtOAc:Hexane gradient starting at 25% EtOAc as eluent to obtain the product (**49)**, a yellow crystalline solid in a yield of 56% (0.65 g). HRMS: calculated mass for $C_{15}H_{21}N_2O_3S$ [M+H] 309.1273; found 309.1277 *m/z*. ATS FT-IR cm^{-1}: 682, 734, 783, 796, 827, 837, 880, 900, 927, 947, 1058, 1072, 1110, 1145, 1161, 1197, 1231, 1314, 1355, 1404,

1451, 1462, 1480, 1588, 2788, 2828, 2929, 3157, 3496. The formation of the product was confirmed by TLC analysis.

Product **49** (0.31 g, 1.0 eq, 1.0 mmol) and imidazole (0.10 g, 1.5 eq, 1.5 mmol) was added to DCM (2.0 mL) at 0 °C under N_2. Triphenyl phosphine (0.39 g, 1.5 eq, 1.5 mmol) was added dropwise over ten minutes. Iodine (0.39 g, 1.5 eq, 1.5 mmol) was added to reaction mixture. The reaction was stirred for one hour at 0 °C. The reaction was quenched with saturated sodium thiosulfate (2.0 mL) and DCM(5.0 mL). The product was extracted with DCM (2 ×15 mL) and concentrated *in vacuo* and purified using column chromatography with EtOAc:Hexane gradient starting at 25% EtOAc was used as eluent to obtain the product(**50**), a yellow oil in a yield of 93% (0.39 g). $C_{15}H_{20}N_2O_2S$ [M+H] 419.0290; found 419.0305 *m/z* for intermediate **50**.

Compound **50** (0.19 g, 1.0 eq, 0.44 mmol) was reacted with sodium azide (0.030 g, 1.0 eq, 0.44 mmol) in DMF for 5 hours starting at 0 °C and warming to room temperature under N_2. The reaction was quenched by dissolution and the addition of water (5.0 mL). The product was dried in *vacuo* at low temperature. The product was purified using flash chromatography with DCM: MeOH as elute. A pale-yellow oil, **41**, was obtained in a yield of 100% (0.15 g). 1H NMR (400 MHz, $CDCl_3$) δ 8.56 (d, *J* = 8.5 Hz, 1H, Ar), 8.30 – 8.22 (m, 2H, Ar), 7.56 (m, 2H, Ar), 7.20 (d, *J* = 7.5 Hz, 1H, Ar), 4.87 (t, *J* = 6.3 Hz, 1H, -$\underline{NH}CH_2$), 3.26 (t, *J* = 6.4 Hz, 2H, -$CH_2\underline{CH_2}N_3$), 2.98 (dd, *J* = 6.4 Hz, 2H, -NH$\underline{CH_2}CH_2$-), 2.89 (s, 6H, $N(CH_3)_2$), 1.66 (p, *J* = 6.5 Hz, 2H, -$CH_2\underline{CH_2}CH_2$-). ^{13}C NMR (101 MHz, $CDCl_3$) δ 152.15, 134.32, 130.64, 129.94, 129.82, 129.52, 128.51, 123.19, 118.45, 115.26, 67.08, 48.77, 45.39, 40.81, 28.79. ATS FT-IR cm^{-1}: 668, 697, 952, 1019, 1310, 1406, 1436, 1661, 2913, 2995, 3441. MP: Oil at room temperature. Mass spectroscopy was not performed, due to 100% conversion of the starting material, confirmed by TLC, IR spectroscopy also indicates the presence of the azide. NMR analysis also compares favorably with those confirmed by literature for compound **40.**

3.3.1.3 *Attempted Synthesis of (S)-2-azidoethyl 2-((tert-Butyldioxycarbonyl)amino)-3-phenylpropanoate (42)*



L-Phenylalanine — N-boc Protected L-phenylalanine — **42**

Scheme 22**: Attempted method used for synthesis of compound **42

d-tert-Butyl dicarbonate (0.22 g, 1.1 eq, 2.4 mmol) was added slowly to l-phenyl alanine(0.20 g, 1.0 eq, 2.2 mmol) and sodium hydroxide (0.20 g, 2.2 eq, 4.9 mmol) and in a water: THF mixture (1:1 ratio, 5.0 mL) and left to stir at room temperature for 12 hours. The reaction was quenched with a saturated sodium chloride solution (5.0 mL). The resultant mixture was concentrated *in vacuo*. The product was then recrystallized from water. HRMS: calculated mass for $C_{14}H_{20}NO_4$ [M+H] 266.1392; found 266.1385 *m/z*.

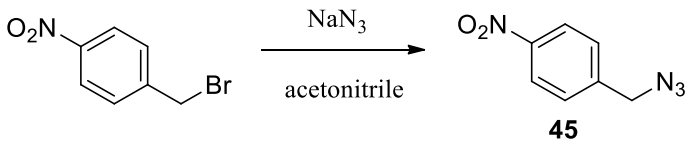
The Boc protected L-phenyl alanine (0.32 g, 1.0 eq, 1.2 mmol) was then dissolved in DMF (5 mL) and sodium hydride (0.03 g, 1.0 eq, 1.2 mmol) and 2-azidoethyl methyl sulfonate (0.20 g, 1.1 eq, 1.2 mmol) were added. This was stirred at room temperature for 20 minutes. The reaction was left to stir at 0 °C for 24 hours. The reaction was quenched with saturated sodium chloride solution (5 mL). The mixture was concentrated under low temperature *in vacuo* and loaded onto silica. The crude material was purified using column chromatography with EtOAc:Hexane gradient starting at 20% EtOAc as eluent to obtain the unidentified, product a white solid in a yield of 95% approx. (0.48 g).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.20 (m, 1H), 7.18 – 7.11 (m, 1H), 4.44 – 4.32 (m, 3H), 4.34 (d, *J* = 4.2 Hz, 1H), 3.88 – 3.75 (m, 1H), 3.70 (s, 1H), 3.63 – 3.55 (m, 2H), 3.08 (s, 4H), 3.08 (s, 1H), 3.00 (s, 1H), 1.40 (s, 4H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 135.77, 129.23, 128.66, 127.18, 71.19, 68.92, 67.43, 67.07, 62.54, 49.85, 37.77, 37.71, 28.25. Unfortunately, NMR spectroscopy indicates that the desired product was not synthesized. These values were compared to literature, indicting the absence of the desired product.[71] Thus, further spectroscopic analysis and identification was not performed. This product was not used in the subsequent click reaction.

3.3.1.4 *Compound 43 and Compound 44*

These compounds were synthesized by a member of the Stellenbosch University Group of Organic and Medicinal Chemistry (GOMOC), Mr. MG Botes, and analysis aligns with expected literature values.

3.3.1.5 *Synthesis of 1-(azidomethyl)-4-nitrobenzene (45)*



45

Scheme 23**: Synthetic procedure used in the production of azide **45

Sodium azide (0.13 g, 1.0 eq, 2.0 mmol) was added to 4-nitobenzyl bromide (0.43 g, 1.0 eq, 2.0 mmol) in acetonitrile (5.0 mL) and stirred at room temperature for 5 hours under N_2. The reaction was quenched with water (10 mL). The product was extracted using ethyl acetate (3 × 15 mL) and washed with a saturated sodium chloride solution (5.0 mL) and concentrated *in vacuo*. The crude material was purified using column chromatography, with an EtOAc:Hexane gradient starting at 5% EtOAc used as eluent, to obtain the product (**45**), a yellow oil in 99% yield (0.36 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (dd, 2H, Ar), 7.54 – 7.46 (dd, 2H, Ar), 4.50 (s, 2H, -$\underline{CH_2}N_3$). Confirmed by literature and TLC analysis.[66]

3.3.2 Click reactions

The following section discusses the synthetic methods used to produce alkyne azide cycloaddition (click) products from the 4*N*-functionalized-aza-podophyllotoxin products **36a** and **36b,** synthesized in Library 1. This section will highlight the optimizations performed, as well as report the best yields obtained. Two general methods were used for the synthesis of the desired products, method A, without the use of a ligand and method B, using a THTPA ligand. Each method included a mild reducing agent, a copper catalyst, the specified azide, and either starting material **36a** or 3**6b**.

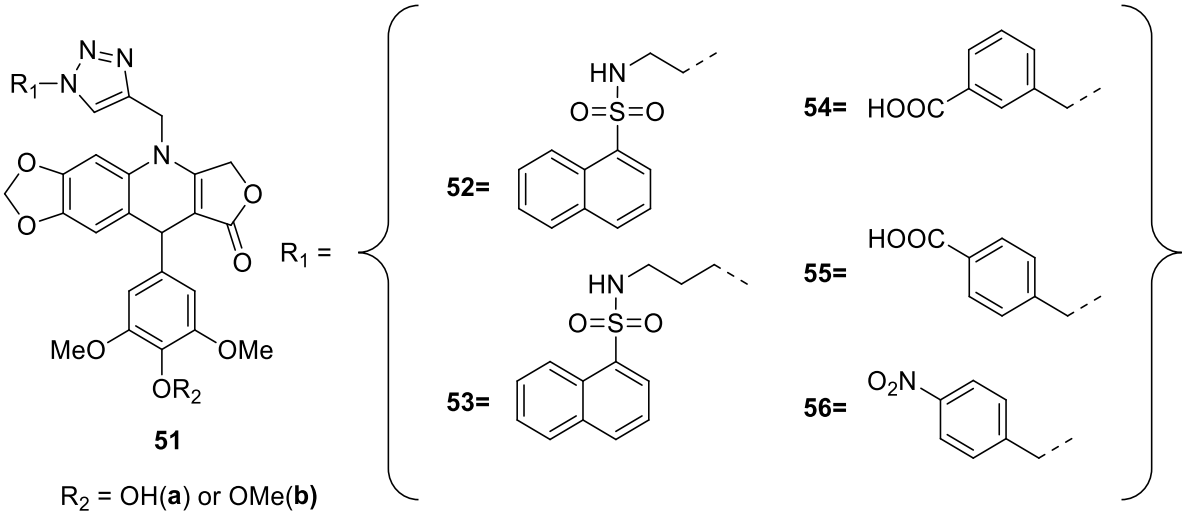


Figure 32: *4-Aza-podophylltoxin analogues attempted through click chemistry of azides* ***40-45*** *with products* ***36a*** *an* ***36b***

3.3.2.1 *Synthesis of products 52a-56b*

This section is split into six parts, each part comprising of one of the click products. All products that were successfully made were synthesized using the general procedure (A). Each section also contains a table displaying the procedures attempted in that section. The products that are marked with an asterisk (* in tables) made use of procedure B. Where solvent mixtures have been indicated in the tables it should be assumed to be in a 1.3:1 ratio, unless otherwise stated. NMR analysis for this section will define peaks as Az (azide peaks) and Pod (Podophyllotoxin core structure).

3.3.2.2 *General procedures A*

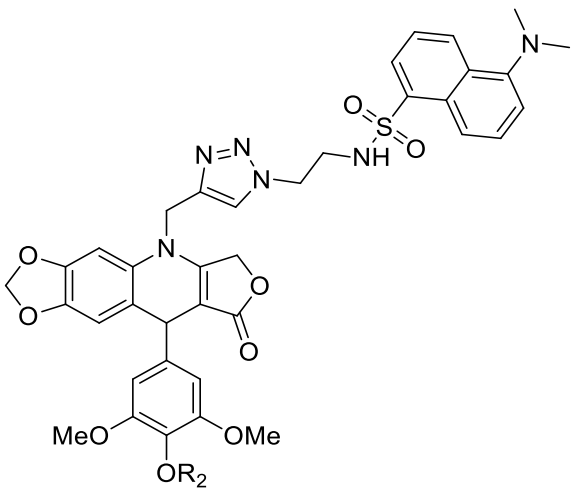
Azide **40-45** (1.0 eq, 0.1 mmol) and the copper catalyst (79 mg, 0.050 eq, 0.050 mmol) were added to a solvent (3.0 mL) at RT and allowed to dissolve. Product **36a/b**, (1.0 eq, 0.10 mmol) and sodium L-ascorbate were added (amount specified in **Tables 10-14)**. The reaction was then stirred for 36 hours, after which the reaction was quenched with a saturated NH_4Cl solution (2.0 mL) and water (1.0 mL). The resultant precipitate was obtained through filtration and washed with ethanol. The precipitate was dried *in vacuo*.

3.3.2.3 *General procedure B*

Prior to setting up the reaction, the "click"-precatalyst, $CuSO_4$/THPTA, was pre-chelated for 10 minutes by mixing aq. $CuSO_4.5H_2O$ (20 µL, 8.0 µmol, 0.40 M, 0.050 eq) and aq. THPTA (40 µL, 8.0 µmol, 0.20 M, 0.050 eq). Starting material **36a/b** (0.10 mmol, 1.0 eq) and azide **40-45** (0.10 mmol, 1.0 eq) were dissolved in solvent (2.0 mL) and added to the reaction flask. Then the $CuSO_4$/THPTA solution (60 µL, 8.0 µmol, 0.13 M, 0.050 eq) was added. Lastly, aq. sodium L-ascorbate (40 µL, 32 µmol, 0.80 M, 0.20 eq) was added to the reaction mixture and it was stirred under N_2 at room temperature for 36 hours. The reaction was next quenched with a saturated NH_4Cl solution (2.0 mL) and water (1.0 mL). The resultant precipitate was obtained through filtration and washed with ethanol. The precipitate was dried *in vacuo.*

The compound characterizations and reaction conditions that were variable for each individual product are shown below.

3.3.2.4 *Synthesis of 52: 4N-aza-posophyllotoxin with fluorescent marker 40*



R_2= -OH (**a**) or -OMe (**b**)

52

***Figure 33**: Expected structure of compound **52** synthesized from **36a/b** and Azide **40**.*

The best crude yield obtained was 55% (41 mg). No pure recovery of starting material or final product was achieved.

Table 10**: Procedures used in the attempted synthesis of fluorescent marker linked 4-aza-podophyllotxin **52

Click Product	Solvent	Time in hours	Temperature	Catalyst(s) (5%)	Amount of sodium L-ascorbate (in eq.)
52a	DMSO	24	RT	$Cu(OAc)_2$	0.1
52a	DMF	24	RT	$Cu(OAc)_2$	0.1
52a	*t*-BuOH: H_2O	24	RT	$Cu(OAc)_2$	0.1
52a	*t*-BuOH: H_2O	24	RT	$Cu(SO_4)\cdot 5H_2O$	0.1
52a	DCM: H_2O	6	RT	$Cu(SO_4)\cdot 5H_2O$	0.1
52a	DMSO	24	60	$Cu(OAc)_2$	0.3
52b	DMSO	24	RT	$Cu(OAc)_2$	0.1
52b	DMSO	32	RT	$Cu(OAc)_2$	0.1
52b	DMSO	12	RT	$CuSO_4$	0.1
52b	DMF	24	RT	$CuSO_4$	0.1
52b	DCM: H_2O	6	RT	$Cu(OAc)_2$	0.1
52b	DMSO	24	60	$Cu(OAc)_2$	0.3
52a*	**DMSO**	**24**	**RT**	**$Cu(SO_4)\cdot 5H_2O$**	**0.2**
52b*	DMSO	24	RT	$Cu(SO_4)\cdot 5H_2O$	0.2

*3.3.2.4.1 Compound **52a***

Due to the impure nature of this product integration of the peaks for this spectrum are not consistent. Integration of this NMR indicates an overlap between peaks of the starting material and the final structure, in a minimum of a 1:5 final product to starting material ratio.

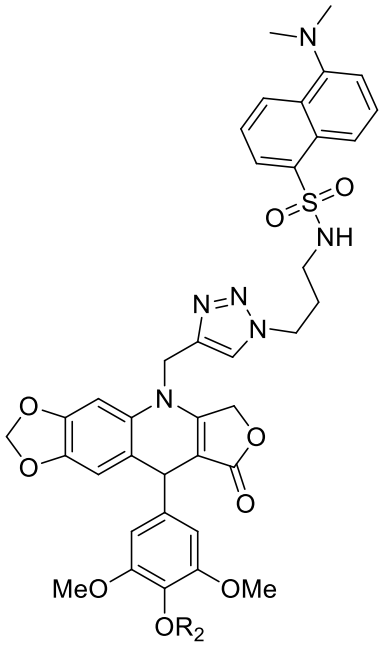
^{1}H NMR (600 MHz, DMSO-d_6) δ 8.68 – 7.10 (m, 1H, Ar, Az), 7.01 – 6.92 (s, 1H, Ar, pod), 6.78 – 6.71 (s, 1H, Ar, Pod), 6.44 – 6.41 (s, 1H, Ar, Pod), 6.08 – 5.91 (dd, 1H, Ar, Pod), 5.24 – 5.13 (d, 1H, Pod), 5.04 – 4.92 (m, 1H, Pod), 4.63 – 4.58 (dd, 1H, Pod), 4.36 – 4.32 (t, 3H, Pod), 3.69 – 3.66 (s, 3H, Pod), 3.48 – 3.38 (qd, 15H, Pod), 2.85 – 2.79 (s, 1H, Az, $N(CH_3)_2$), 1.85 (t, 0.1H, Az, $\underline{CH_2}CH_2N$) 1.08 – 1.03 (t, 31H, Pod). ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.27, 159.57, 153.28, 147.32, 144.09, 142.73, 136.55, 131.09, 119.75, 110.27, 104.92, 101.98, 97.68, 97.07, 78.73, 76.16, 65.67, 60.33, 56.49, 56.28, 40.88, 36.12, 19.02.

IR spectrum showed insufficient material present at the time or testing. HRMS: calculated mass for $C_{37}H_{37}N_6O_9S$ [M+H] 741.2343; found 741.2325 *m/z*. MP 184-186 °C.

*3.3.2.4.2 Compound **52b***

Compound **52b** was not observed with NMR spectroscopy and the starting material was exclusively recovered. Recovery of 96% starting material (**36b**) was achieved (42 mg).

3.3.2.5 ***Compound 53: 4N-aza-podophyllotoxin with fluorescent marker 41***



R_2= -OH (**a**) or -OMe (**b**)

53

***Figure 34**:* Desired product **53** for the click reaction between compound **41** and **36a/b**

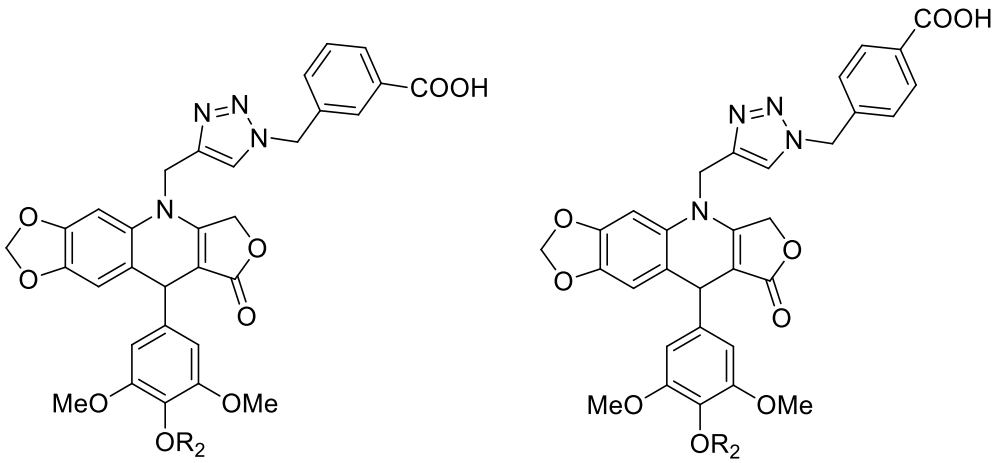
No yield of desired product was obtained, recovery of 98% of starting material (**36a/b**) was achieved.

Table 11**: Methods used in the synthesis of products **53a** and **53b

Click Product	Solvent	Time in hours	Temperature	Catalyst(s) (5%)	Amount of sodium L-ascorbate(in eq.)
53a	DMSO	24	RT	$Cu(OAc)_2$	0.1
53a	DMF	24	RT	$Cu(OAc)_2$	0.1
53a	*t*-BuOH: H_2O	24	RT	$Cu(OAc)_2$	0.1
53a	*t*-BuOH: H_2O	24	RT	$Cu(SO_4)\cdot 5H_2O$	0.1
53a	DCM:H_2O	6	RT	$Cu(SO_4)\cdot 5H_2O$	0,1
53a	DMSO	24	60	$Cu(OAc)_2$	0.3
53b	DMSO	24	RT	$Cu(OAc)_2$	0.1
53b	DMSO	32	RT	$Cu(OAc)_2$	0.1
53b	DMSO	12	RT	$Cu(SO_4)\cdot 5H_2O$	0.1
53b	DMF	24	RT	$Cu(SO_4)\cdot 5H_2O$	0.1
53b	DCM: H_2O	6	RT	$Cu(OAc)_2$	0,1
53b	DMSO	24	60	$Cu(OAc)_2$	0.3

No product was observed in ^{1}H NMR spectrum despite multiple attempts. Starting materials **36a/b** and **41** was exclusively recovered.

3.3.2.6 *Compound 54: 4N-aza-podophyllotoxin with meta-benzylic acid*



R_2= -OH (**a**) or -OMe (**b**)
54

R_2= -OH (**a**) or -OMe (**b**)
55

***Figure 35**: Structures of compounds **54** and **55** synthesized from **36a/b** and azides **43** and **44**.*

Table 12: *Methods attempted in the synthesis of* ***54a*** *and* ***54b***

Click Product	Solvent	Time	Temperature	Catalyst(s)	Amount of sodium L-ascorbate (in eq.)
54a	DMSO	24	RT	$Cu(OAc)_2$-5%	0.1
54a	DMSO	24	60	$Cu(OAc)_2$-5%	0.15
54a	DMSO	48	RT	$Cu(OAc)_2$-10%	0.5
54a	**DMSO**	**48**	**60**	**$Cu(OAc)_2$-10%**	**0.2**
54b	DMSO	24	40	$Cu(OAc)_2$-10%	0.3

3.3.2.6.1 Compound ***54a***

The best yield obtained was 68%. (41 mg).

^{1}H NMR (300 MHz, DMSO) δ, 9.12 (s, 1H, OH), 8.38-8.36 (m, *J* = 1.9 Hz, 1H, Az, Ar), 8.16 (ddd, *J* = 8.1, 2.4, 1.1 Hz, 1H, Ar, Az), 8.06-8.03 (m, *J* = 7.8, 1.3 Hz, 1H, Ar, Az), 7.75 (t, *J* = 7.9 Hz, 1H, Ar, Az), 7.11 (s, 1H, Ar, Pod), 6.74 (s, 1H, Ar, Pod), 6.47 (s, 2H, -OCH_2-, Pod), 6.06 – 5.85 (m, 2H, Ar, Pod), 5.48 – 5.26 (m, 1H, Pod), 5.21 (d, *J* = 16.6 Hz, 1H, Pod), 4.98 (d, *J* = 16.9 Hz, 1H, Pod), 4.86 (s, 1H, Pod), 3.86 – 3.67 (m, 1H, Pod), 3.64 (s, 6H, Pod), 3.56 (s, 2H, Pod),2.09 (s, 2H, Az, NCH_2-Ar). HRMS: calculated mass for $C_{31}H_{27}N_4O_9$ [M+H] 599.1778, found 599.1768 *m/z*. ATS FT-IR cm^{-1}: Suggests degradation of synthesized product. MP 204-206°C.

3.3.2.6.2 Compound ***54b***

The compound was not synthesized in sufficient quantities for further analysis. <10% yield (approx. 4 mg)

3.3.2.7 *Compound 55: 4N-aza-podophyllotoxin with para-benzylic acid*

Table 13: *Methods used in the attempted synthesis of* ***55a*** *and* ***55b***

Click Product	Solvents	Time	Temperature	Catalyst(s) 5% or 10%	Amount of sodium L-ascorbate (in eq.)	Yield in %
55a	DMSO	24h	RT	$Cu(OAc)_2$	0.1	-
55a	*t*-BuOH: H_2O	24h	RT	$Cu(SO_4)\cdot 5H_2O$	0.3	-
55a	DMSO	24h	RT	$Cu(OAc)_2$	0.1	-
55b	DMSO	24h	40	$Cu(OAc)_2$	0.1	-
55b	DMSO	24h	RT	$Cu(OAc)_2$	0.3	10
55b	DMSO	24h	60	$Cu(OAc)_2$-10%	0.5	-
55a*	***t*-BuOH: H_2O**	**24h**	**RT**	**$Cu(SO_4)\cdot 5H_2O$**	**0.2**	**65**
55b*	*t*-BuOH: H_2O	24h	RT	$Cu(SO_4)\cdot 5H_2O$	0.2	20

3.3.2.7.1 Compound ***55a***

Initially it was thought that 65% yield (39 mg) was obtained, based on NMR spectroscopy, however mass spectroscopy did not correlate with the expected value. This suggests that what was thought to be the final product was actually a 1:1 mixture of the starting materials, or that an unknown reaction to form a side product took place.

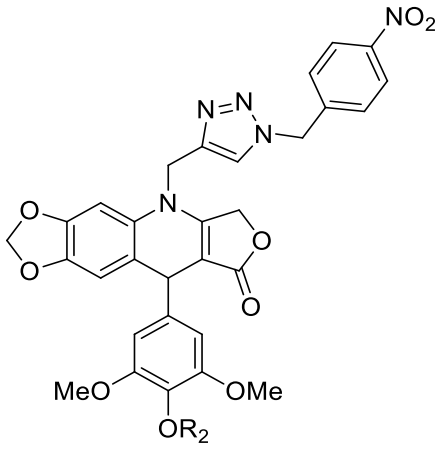
^{1}H NMR (600 MHz, DMSO-d_6) δ 8.40 – 8.30 (s, 1H, Ar, Az), 8.20 – 8.05 (m, 2H, Ar, Az), 7.81 – 7.69 (t, *J* = 7.5 Hz, 1H, Ar, Az), 7.16 – 6.98 (d, *J* = 63.5 Hz, 1H, Ar, Pod), 6.79 – 6.69 (d, *J* = 34.7 Hz, 1H, Ar, Pod), 6.48 – 6.34 (d, *J* = 6.6 Hz, 2H, -OCH_2O-), 6.07 – 5.81 (m, 2H, Ar Pod), 5.41 – 5.35 (d, *J* = 15.8 Hz, 1H, Pod), 5.29 – 5.16 (dd, *J* = 37.3, 16.1 Hz, 2H, pod), 5.01 – 4.94 (dd, *J* = 16.4, 9.0 Hz, 1H, Pod), 4.84 – 4.70 (d, *J* = 3.7 Hz, 1H, Pod), 4.67 – 4.56 (m, 1H, Pod), 3.74 – 3.66 (s, 2H, Pod), 3.68 – 3.56 (s, 6H, Pod), 2.12 – 2.05 (s, 2H, Az), 2.00 – 1.97 (s, 1H, Pod), 1.19 – 1.14 (t, *J* = 7.1 Hz, 1H, Pod). ^{13}C NMR (101 MHz, DMSO-d_6) δ 206.69, 171.98, 170.54, 159.30, 153.01, 147.06, 143.82, 142.45, 136.28, 130.83, 119.47, 109.99, 104.66, 101.71, 97.41, 96.80, 78.46, 75.90, 65.39, 62.99, 60.06, 59.95, 56.01, 35.85, 30.89, 20.96, 14.28.HRMS: calculated mass for $C_{30}H_{23}N_4O_9$ [M+H] 583.1465 -found 583.1464 *m/z*. ATS FT-IR cm^{-1}:

685, 705, 756, 791, 828, 874, 935, 1015, 1044, 1112, 1203, 1238, 1276, 1320, 1361, 1434, 1477, 1508, 1608, 1643, 1726, 2114, 2900, 3246. MP:190-192 °C.

*3.3.2.7.2 Compound **55b***

A mixture of products was present in the mass spectrum. Peaks appeared at 871.2703 *m/z*, and 436.1391 *m/z*. These values correspond with THPTA (2M+H) and the starting material, compound **36b**.

3.3.2.8 *Compound 56: 4N-aza-podophyllotoxin with 4-nitrobenzene*



R_2= -OH (**a**) or -OMe (**b**)

56

***Figure 36**: Structure of product **56** from azide **45** and 4-aza-podophylltoixn derivatives **36a/b**.* The best yields obtained were 97% (58 mg) for **56a** and 65% (40mg) for **56b**

Table 14**: Methods used to synthesize **56a** and **56b

Click Product	Solvents	Time	Temperature	Catalyst(s) (5%)	Amount of sodium L-ascorbate (in eq.)	Yield in %
56a*	*t*-BuOH: H_2O	24h	RT	$Cu(SO_4)\cdot 5H_2O$	0.2	75
56a*	**DMSO**	**24h**	**RT**	**$Cu(SO_4)\cdot 5H_2O$**	**0.2**	**97**
56b*	*t*-BuOH: H_2O	2h	RT	$Cu(SO_4)\cdot 5H_2O$	0.2	65
56b*	**DMSO**	**24h**	**RT**	**$Cu(SO_4)\cdot 5H_2O$**	**0.2**	**78**

*3.3.2.8.1 Compound **56a***

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (s, 1H, OH), 8.25 – 8.11 (m, 2H, Ar, Az), 8.09 (s, 1H, Az, Ar), 7.53 – 7.44 (m, 2H, Ar, Az), 7.02 (s, 1H, Ar, Pod), 6.69 (s, 1H, Ar, Pod), 6.40 (s, 2H, -OCH$_2$O-), 5.94 (s, 1H Ar, Pod), 5.92 – 5.84 (m, 1H, Pod), 5.76 (s, 2H, Pod), 5.28 (d, *J* = 15.9 Hz, 1H, Pod), 5.17 (d, *J* = 15.7 Hz, 1H, Pod), 5.08 (d, *J* = 16.8 Hz, 1H, Pod), 4.87 (d, *J* = 17.0 Hz, 1H, Pod), 4.75 (s, 1H), 3.62 (s, 6H), 3.69 – 3.52 (m, 1H), 2.02 (s, 2H, Az), 1.15 (q, *J* = 6.3, 5.5 Hz, 1H, Pod). ^{13}C NMR (75 MHz, DMSO-d_6) δ 208.63, 206.81, 172.27, 170.52, 159.61, 147.92, 147.75, 147.35, 146.72, 143.39, 142.64, 137.51, 134.20, 130.63, 129.29, 129.14, 128.85, 124.38, 124.05, 123.98, 123.96, 119.85, 110.06, 104.85, 101.43, 96.39, 96.28, 68.64, 65.78, 59.88, 56.19, 56.14, 55.99, 55.98, 55.89, 52.04, 48.69, 32.20, 30.78, 29.66, 20.85, 14.17.HRMS: calculated mass for $C_{30}H_{26}N_5O_9$ [M+H] 600.1731-found 600.1743 *m/z*. ATS FT-IR cm^{-1}: 686, 731, 791, 859, 930, 1037, 1113, 1199, 1241, 1276, 1354, 1426, 1473, 1508, 1649, 1727, 2359, 2941, 3484. MP: 196-198 °C.

*3.3.2.8.2 Compound **56b***

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.32 (s, 1H, Ar, Az), 8.27 – 8.16 (m, 2H, Ar Az), 7.48 (d, *J* = 8.4 Hz, 1H, Ar, Az), 7.01 (d, *J* = 19.8 Hz, 1H, Pod), 6.75 (d, *J* = 21.9 Hz, 1H, Pod), 6.46 (s, 2H, Pod), 6.03 – 5.85 (m, 2H, Pod), 5.76 (s, 1H, Pod), 5.34 – 5.19 (m, 1H, Pod), 5.19 – 5.05 (m, 1H, Pod), 4.91 (m, 1H, Pod), 4.82 (d, *J* = 5.6 Hz, 1H, Pod), 4.69 – 4.50 (m, 1H, Pod), 3.66 (d, *J* = 14.4 Hz, 6H, Pod), 3.57 (d, *J* = 6.9 Hz, 3H, Pod), 3.31 (s, 2H, Pod), 2.06 (s, 1H, Az), 1.97 (s, 1H, Az), 1.15 (t, *J* = 7.1 Hz, 1H, Pod). ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.56, 172.29, 170.80, 159.93, 159.35, 148.36, 148.30, 147.73, 147.18, 147.10, 144.01, 143.79, 143.75, 143.02, 137.85, 137.39, 134.78, 134.60, 131.05, 131.03, 129.64, 129.50, 124.74, 124.43, 124.36, 120.21, 110.43, 110.28, 105.26, 101.91, 101.79, 97.93, 96.96, 96.77, 96.66, 78.76, 76.13, 66.13, 60.22, 56.49, 56.46, 56.40, 56.36, 52.39, 36.09, 31.15, 21.51, 21.22, 19.02, 14.55. HRMS: calculated mass for $C_{31}H_{28}N_5O_9$ [M+H] 614.1887-found 614.1883 *m/z*. ATS FT-IR cm^{-1}: 686, 707, 732, 764, 790, 813, 836 ,863, 925, 975, 925, 975, 1020, 1046 , 1125, 1201, 1236, 1350, 1423, 1476, 1503, 1525, 1588, 1611, 1647, 1727, 2107, 2940, 3219. MP: 196-198 °C.

Chapter 4 Biological activity

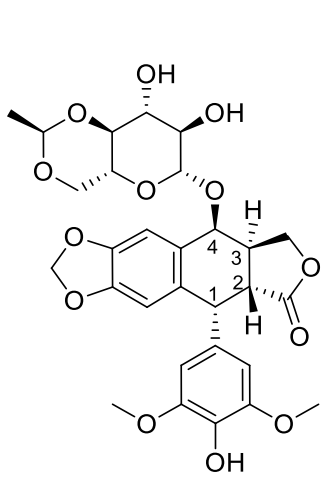
4.1 Introduction

(-) Podophyllotoxin, the naturally occurring cyclolignan, has shown significant biological activity against a number of diseases, its most significant use is as an anti-cancer agent. Seven of its semisynthetic analogues have made it into clinical trials or are currently in use as anticancer agents.[1] These are etoposide (**9**), teniposide (**10**), etopophos (currently in use[1]), NK-611 (**12**), GL331 (**14**), NPF (**13**), and TOP53 (**15**) (currently in clinical trials, see **Figure 37**).[1] Etoposide (aka Vepesid®, **9**), its prodrug etopophos (**11**), and teniposide (**10**) are commercially used and approved by the FDA as anticancer agents.[6,36] Podophyllotoxin and its analogues have also been approved as antivirals (mainly for treatment of the human papilloma virus (HPV), in dermatology (for the treatment of venereal warts), as vascular disrupting agents, and as an arthritis medication.[27] Podophyllotoxin analogues have been extensively used to treat Wilms tumors, non-Hodgkin and other lymphomas, colon and lung cancer.[17]

The semi-synthetic analogues (**9-15**) all show the same stereomeric form at C1 form which has been found to be the more active of the two enantiomers (the R enantiomer is active, while the S is not with regards to the C1 position, see **Figure 37**, structure **9**). The stereochemistry of the C4 position in the biologically active semi-synthetics is opposite to that of naturally occurring (-)-podophyllotoxin.[1] Research done by Magedov *et al.*[8] found that the optical antipode of 4-aza-podophylltoxin analogues formed through MCR, that match naturally occurring podophyllotoxin show significantly heightened biological activity when compared to their S enantiomeric counterpart in relation to the C1 position.[17] That said, there is little evidence suggesting that a mixture of the two diastereomers will lead to either synergistic or anti-synergistic effects. Therefore, in this project the individual isomers were not isolated, and a mixture of the enantiomers was used in the subsequent biological testing.

Natural podophyllotoxin and etoposide show IC_{50} values of between 0.076 and 0.05 µM against HeLa and KB cell lines, which is similarly reported for the 4-aza-podophyllotoxin analogues.[1] Detailed information on the various IC_{50} values have been reviewed by Yu et al.(2017).[1] For the 4-aza-podphylltoxin analogues, IC_{50} values of below 3 µM were obtained against several cell lines, with most being in nM range

against HeLa, B16, A549 and other cell lines.[1] There have not been any reported cases of testing against esophageal and other cell lines. This motivated the biological testing, the results of which are presented in this chapter.



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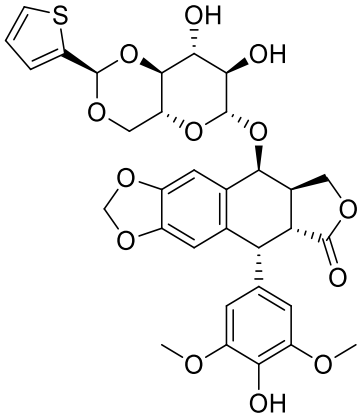

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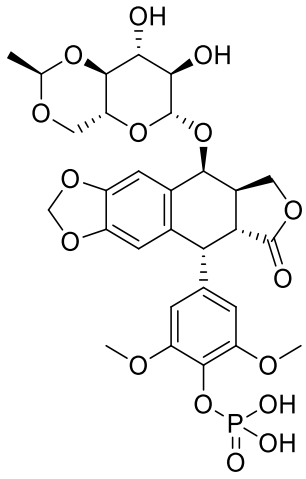

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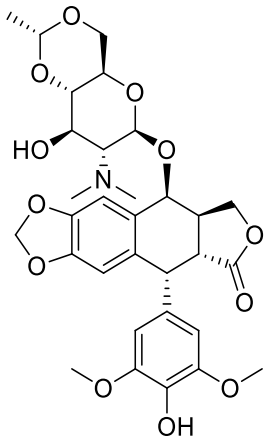

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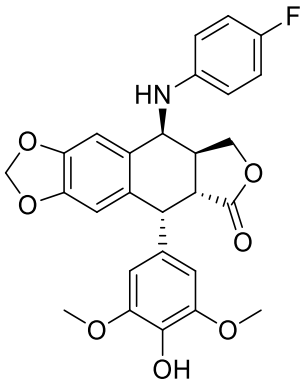

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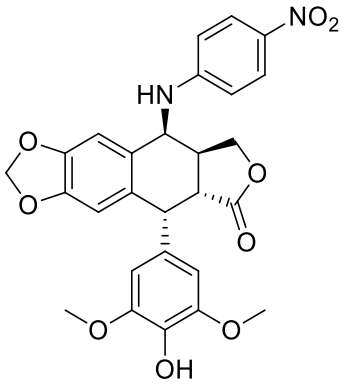

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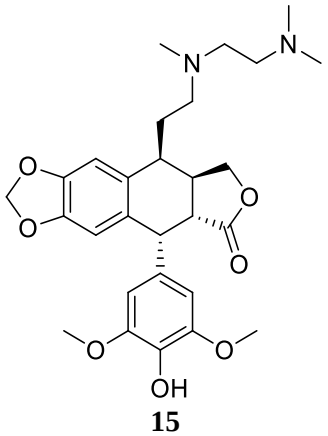

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***Figure 37**: The structures of Etoposide (**9**), Teniposide (**10**), Etopophos (**11**), NK-611(**12**), NFP (**13**), GL331(**14**) and TOP53 (**15**)*

The experiments undertaken in this part of the project were focused on compounds synthesized in the first library, specifically: **33a/b**, **34a**, **35a**, **36a/b**, **37a/b** and **38a/b**, shown in **Figure 38** below.

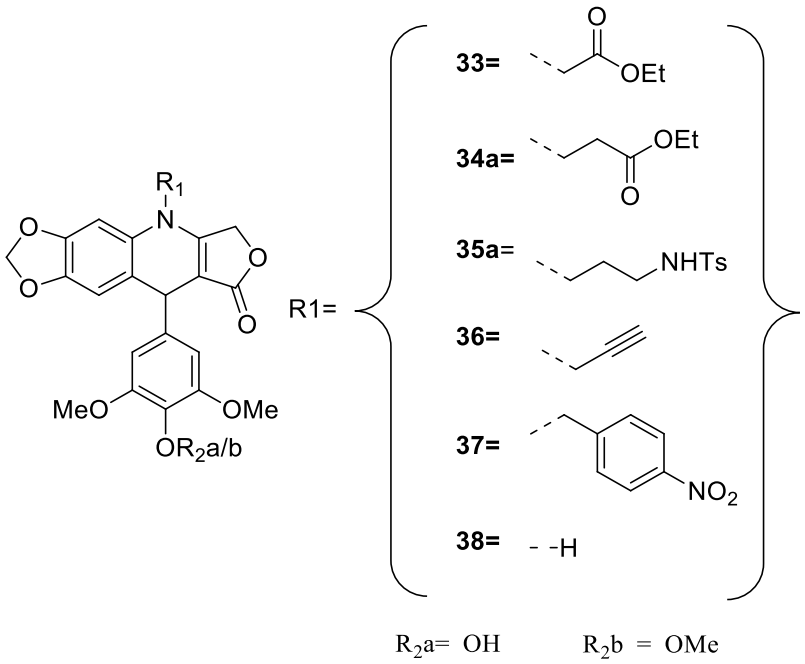


***Figure 38**: The compounds sent for biological testing, all from Library 1.* [*3]

Compound **35a** has been included in the set of compounds that were biologically tested despite their lack of correlation with literature-based analysis of ^{1}H NMR spectroscopy and mass spectral data.

This chapter will begin by outlining the biological testing performed by the author with the assistance of the GOMOC and the Stellenbosch University Department of Physiology under the direction of Dr CH Kaschula. It then presents the preliminary testing undertaken by our collaborators at Youngstown State University (YSU). This is followed by a section outlining the experiments performed using MTT cell line assays in the Department of physiology at Stellenbosch University. It concludes by presenting the

*3 These compounds were tested both by our group (GOMOC) and our collaborators in the USA.

summarized findings. The IC_{50} values reported in this chapter come from at least three data sets, each run-in triplicate. The data sets have also been modified to exclude outlying data points.

4.2 Results and discussion

4.2.1 Testing by GOMOC

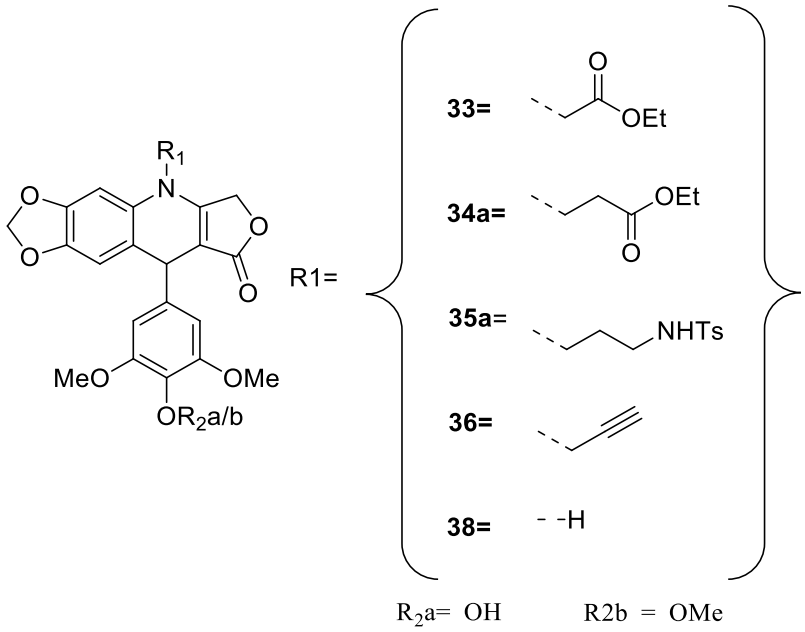
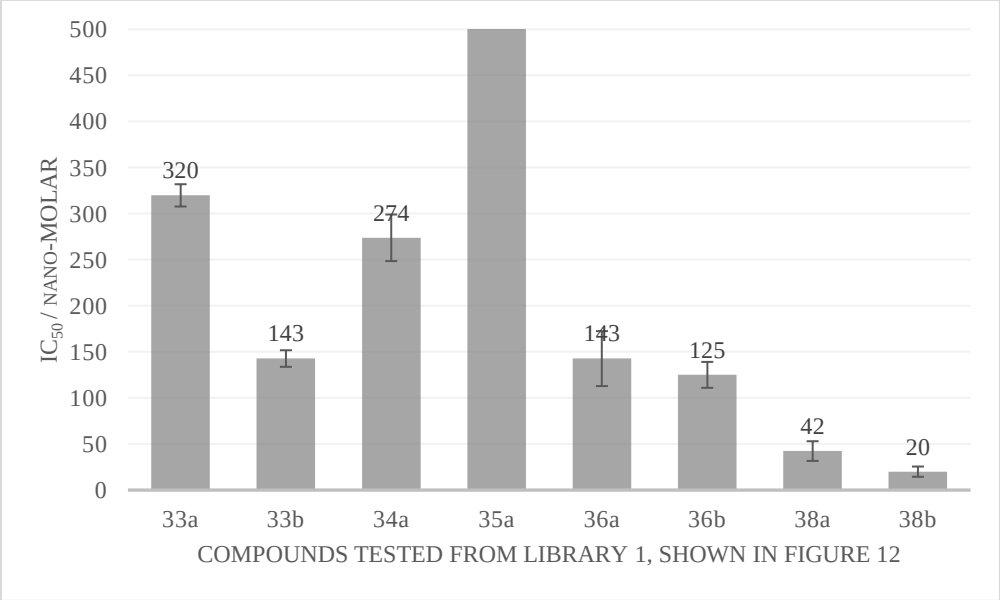


***Figure 39**: Products tested by GOMOC against the WHCO1 cell line assay*[*4]

The biological testing of the of compounds shown in **Figure 39** present a clear trend; compounds with the methoxy functionality in the R_2 position were more biologically active than those with phenolic functional group. This is most likely be due to an interaction between the tubulin binding site and lower E ring. We further stipulate that the methoxy functionality binds more efficiently to the phenolic moiety.[1] The IC_{50} values obtained for the WHCO 1 cell line also indicate that the unfunctionalized compounds (**38a/b**) show the greatest activity. Compounds **38a** and **38b**, displayed IC_{50} values of 42 ± 20 nM and 20 ± 11 nM respectively. These compounds were found to be significantly more active. The results of the preliminary

*4 The compounds from Library 1 not shown in Figure 21 were not synthesized in time for biological testing i.e. compounds **36a/b** and **37a/b.**

testing fall within the nM IC_{50} value ranges observed by Magedov *et al.* for the unfunctionalized 4-aza-podophyllotoxin compounds.[8]



Graph 1: *IC_{50} values obtained for the compounds tested from Library 1 (**see Figure 39**). Values obtained from GOMOC, tested against a WHCO1 esophageal cell line assay.*

The compounds with propargyl groups in the R_1 position (**36a** and **36b**) were the next most active compounds, with IC_{50} values of 143 ± 60 and 125 ± 28 nM. Those with ester functionalities showed the second lowest activity, though still in the nM range (**33a**, **33b** and **34a)**. Of the three, the lowest IC_{50} value was for **33b** with an IC_{50} of 143 ± 18 nM, which falls in the same range as **36a** and **36b**. Finally, compound **35a**, showed little to no activity when compared to the other compounds in this data set. This further justifies the conclusion that compound **35a** does not conform to the 4-aza-podophylltoxin structure. The moderate activity of compounds **36a** and **36b** inspired the further generation of compounds with a triazole functionality (Library 2) via a click reaction approach. In our opinion, the propargyl groups also showed the best potential for further functionalization in a way that would not impact the core 4-aza-podophyllotoxin structure. The results were within our expectation, with biological activity in a >500 nM range which is sufficient to prove significant anti-cancer activity.

4.2.2 **Preliminary testing by Youngstown State University**

The following preliminary results were obtained from our YSU collaborators in the USA. Compounds from library 1 were tested against a skin (SK-Mel-28), a lung (A549), and two breast (MDA-MB-231 and MCF7) cancer cell lines.

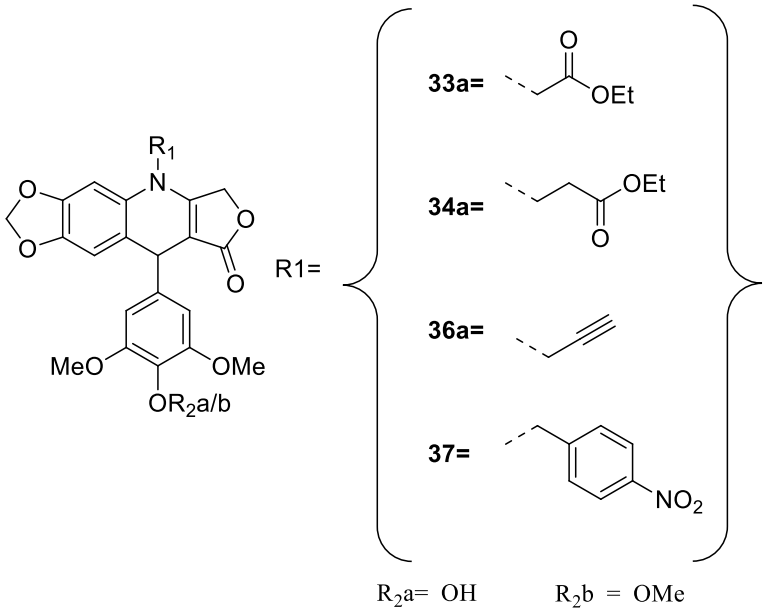


***Figure 40**: Products from Library 1 tested by our collaborators at YSU.*

***Table 15:** IC_{50} raw data obtained from YSU for compounds **33a**, **34a**, **36a**, **37a**, **37b;** naturally-occurring podophyllotoxin and anticancer agent camptothecin, when tested against cell line assays.*

Product	**IC_{50} values obtained in nM**			
Cell line:	**MDA-MB-231**	**SK-MEL-28**	**MCF7**	**A549**
33a	27±24.0	29.7±24.2	>100	>100
34a	6.6±3.2	12.7±6.4	>100	>100
36a	<10	7.3±0.6	>100	>100
37a	-	10-50*	>100	>100
37b	18.5 ± 12.021	10-100*	>100	>100
Podophyllotoxin	<10	<10	~ 10	~ 10
Camptothecin	<10	<10	~10	~10

Despite the compounds from **Figure 40** having been sent to YSU in April 2019, by December the results obtained were unfortunately preliminary in nature (see **Table 15**). Therefore, no firm predictions

can be made from this data set. This data set does appear to follow the general trend obtained by GOMOC. The YSU data set shows that compound **36a** is, on average, comparatively more active than compound **33a** in the SK-MEL-28 cell line. From the table, compound **34a** is slightly more active than compound **33a**, as was observed in the previous data set.

The IC_{50} values were observed when comparing **33a** and **34a** do not indicate improved selectivity of the longer chain ester moiety for the cell lines SK-MEL-28 and MDA-MB-231, due to the preliminary nature of the testing, but do indicate superior activity is exhibited by **34a**. Compounds **33a** and **34a** do show very slightly improved activity against the MDA-MB-231 cell line compared to the SK-MEL-28 cancer cell line. Lower IC_{50} values are consistently observed for **34a** compared to **33a** over all cell lines, further indicating improved activity for the compounds with an extra CH_2 group (**34a/b**). The GL331 (**14**) inspired compounds **37a** and **37b**, despite not having obtained reproducible data, show biological activity against the SK-MEL-28 cell line, indicated with an asterisk (*) in **Table 15**. Unfortunately, accurate data could not be obtained for the MCF7 and A549 cancer cell lines due to reduced cell viability, with only 55-60% of the cells showing viability, making results inconclusive. The data for these two cell lines do, however, indicate initial activity of a factor of 10 lower than that of the naturally occurring podophyllotoxin. Our collaborators in the USA are currently repeating the experiment, and we hope to have this data in due course. The IC_{50} values for the WHCO1 cancer cell line (ranging from 125-500 nM for the same compounds) were on average higher than those obtained for the SK-MEL-28 and MDA-MB-231 cancer cell lines (ranging from 7-30 nM). These results suggest that focus should be placed on the SK-MEL-28 and MDA-MB-231 cancer cell lines in the future.

4.3 Conclusions

The preliminary biological data shows two significant trends. First, that the compounds from Library 1 show biological activity against a range of cancer cell lines (with the exception of **35a**), with IC_{50} values comparable to naturally-occurring podophyllotoxin, and other podophyllotoxin analogues. Second, those compounds with a methoxy moiety in the R_2 position were on average more active than those with phenolic functionalities. The initial WHCO1 cancer cell line assay results indicate that compounds **38a** and

38b showed the most biological activity. Both data sets also indicate that the ester (**33** and **34)** and propargyl 4*N*-functionalized compounds from Library 1 show similar activity across all cell lines. Finally, compound **33a** is typically less active than **34a**, indicating that lengthening the ester moiety may improve biological activity. Future work will include obtaining reproducible data for all cell lines, with focus being placed on compounds **37a** and **37b** and the SK-MEL-28 and MDA-MB-231 for comparison against GL331 (**14**).

4.4 Experiments performed using the MTT cell line assay

4.4.1 Cell growth conditions

All WHCO1 cells were grown in the laboratory of the Stellenbosch Physiology Department.

The WHCO1 cell line (esophageal cancer cells, of South African origin), was derived from a biopsy of primary esophageal squamous cell carcinoma. Cells were all incubated at 37 °C under 5% CO_2 (the cells were split every three days) and cultured with antibiotics in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (Foetal Bovine Serum, Gibco, Life Technologies, South Africa).

4.4.2 Cell Cytotoxicity Assay

The cytotoxicity of the 4-aza-podophyllotoxin analogues **(33-38)** were quantified using the standard MTT cellular viability assay. Briefly, WHCO1 oesophageal cancer cells were seeded at a density of 2.5×10^{30} cells per well in 90 µL DMEM growth media, supplemented with 10% FBS and 2% penicillin/streptomycin in a 96-well culture dish. The cells were allowed to attach overnight. The following day 10 µL of 2-fold dilutions (0 - 800 nM) of the 4-aza-podophylltoxin analogue (previously specified) in DMSO (0.2% v/v) was added in triplicate to the cells and incubated for 48 hours. Thereafter, 10 µL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) was added and incubated with the cells for 4 h. The resulting formazan crystals were solubilised by adding 100 µL of 10% sodium dodecyl sulphate (Sigma-Aldrich) to each well and incubated overnight at 37 °C. The absorbance at 595 nm was measured using a Multiskan FC multi-well reader and the data was fitted using Graphpad prism v5 using log (inhibitor) vs response (variable slope) from which a cytotoxicity IC_{50} value

was obtained. All compounds were tested independently, more than three times, from which an average value and standard deviation of that average was obtained. [*5]

*5The work done for the experiments for this chapter was split between the author and Dr CH Kaschula, with the assistance of members of GOMOC under the supervision of Dr Kaschua.

Chapter 5 Conclusions and future work

5.1 Conclusions

The aims of this research were first to synthesize a diverse library of modified 4-aza-podophyllotoxin-analogues through a set of multicomponent reactions to further investigate structure activity relationships. The second aim was to add biologically active moieties to the 4-*N* position of the newly synthesized analogues that can selectively target cancer cells, or act as phosphorescent markers. It was also hoped that these modified compounds would hold increased selectivity for cancer cells, while reducing toxicity to human cells, a goal which has not been achieved due to a lack of biological date. The challenges faced during synthesis further contributed to the lack of further data.

Twelve unique 4-aza-podophylltoxin analogues were successfully synthesized for Library 1 from the initially reported 6 *N*-functionalized anilines. Though other anilines were synthesized in this project, they did not yield the desired 4-aza-podophyllotoxin analogues in yields sufficient for analysis. For Library 1 most products were synthesized in yields below 50%. The reactions that made use of microwave irradiation and 4-chloroaniline provided more significant yields. It was also proven that the products from Library 1 can undergo further functionalization without affecting the central 4-aza-podoophylltoxin scaffold. The 4*N*-functionlaised 4-aza-podophylltoxin analogues from Library 1 were all synthesized in yields sufficient for further analysis. Of these 12 compounds, six contained the phenolic group in the R_2 position and six the methoxy moiety, making them ideal for comparative analysis.

The biological data for Library 1 shows two significant trends. First, that the compounds show biological activity against a range of cancer cell lines (with the exception of **35a**), with IC_{50} values in the normal range comparable to naturally occurring podophyllotoxin. Second, those compounds with a methoxy moiety in the R_2 position are on average more active than those with phenolic functionalities. The WHCO1 cancer cell line assay results indicate that compounds **38a** and **38b** show the most biological activity. Both data sets, from YSU and GOMOC, also indicate that the ester (**33** and **34)** and propargyl 4*N*-functionalized compounds form Library 1 show nM activity across all cell lines. Finally, compound **33a** is typically less active than **34a**, indicating that lengthening the ester moiety may improve biological activity.

Four compounds were successfully synthesized for Library 2 but require further purification before biological testing. Of these, three contain the phenolic moiety in the R_2 position and one contains the methoxy moiety in the R_2 position. One analogue was successfully coupled to a fluorescent marker, making it ideal for further biological testing. It was observed that the click reactions which employed THPTA as a ligand performed significantly better than those that did not.

It has been shown that the synthesis of the compounds from Library 1 and 2 is reproducible, making them ideal for further study. Of the compounds synthesized in Library 1, most show biological activity in the nM range against an esophageal cell line, showing activity comparable to naturally occurring podophyllotoxin.

5.2 Future work

Future work will revolve around the synthesis of the missing compounds, **35a/b** from Library 1 and **52b**, **53a**, **53b**, **54b**, **55a** and **55b** from Library 2. The synthesis of **35a** and **35b** will revolve around the optimization of the synthesis of the precursor amine via ethanolamine, or the synthesis of an azide that will result in a terminal amine being produced after the copper catalyzed azide alkyne cycloaddition. For the unsuccessful cycloaddition reactions further exploration into potential reaction conditions will be done i.e. the use of DMSO as well as increasing heat without degrading the starting material. Focus will need to be placed on the compounds from Library 2 as the procedures for the synthesis of these compounds has been thoroughly explored and assist in the generation of a large group of diverse 4-aza-podophylltoxin analogues.

Biological testing of **37a** and **37b** against the SK-MEL-28 and MDA-MB-231 cancer cell lines for comparison against GL331 (**14**) will also be undertaken.

Significant work will need to be done into the isolation of the enantiomers to further support the work done by Magedov *et al*.[8] Improved isolation methods for the products obtained in this project will also need to be developed. Though DCM: methanol, or acetonitrile: water column chromatographic separations are effective for isolation of the MCR products are racemic mixtures, chiral stationary phase chromatography will be required to obtain the isolated enantiomers.

The overall yields of the products obtained (as discussed in **Chapter 2**) were not sufficient for significant biological testing. Therefore, further optimization of the reaction conditions will be required. Exploration of enantiomeric selectivity during the ring closure reaction should also be explored. If it is possible to selectively make the R enantiomer, more accurate biological data can be obtained.

The further modification of compounds **34** and **37** after the MCR should also be further explored, with specific focus placed on the reduction of the nitro moieties without affecting the central scaffold.

Other cell lines should also be investigated to test for selectivity, most importantly the impacts of these agents on healthy cell lines. Confocal microscopy should also be used in order to observe the target site of the fluorescent labeled compounds.

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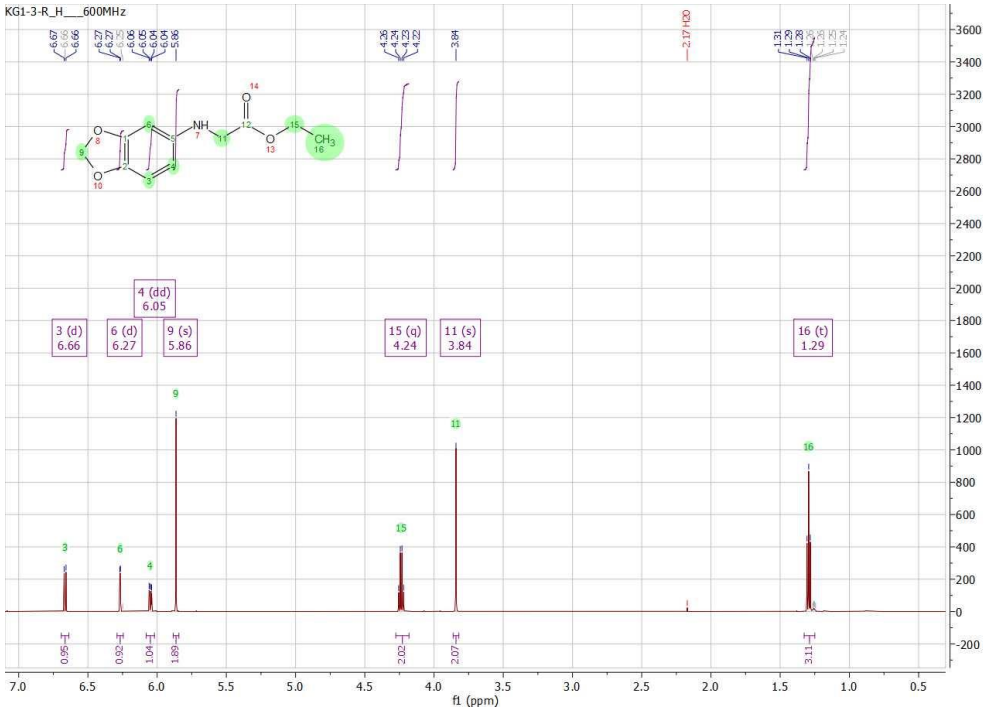
Supplementary information- Part I

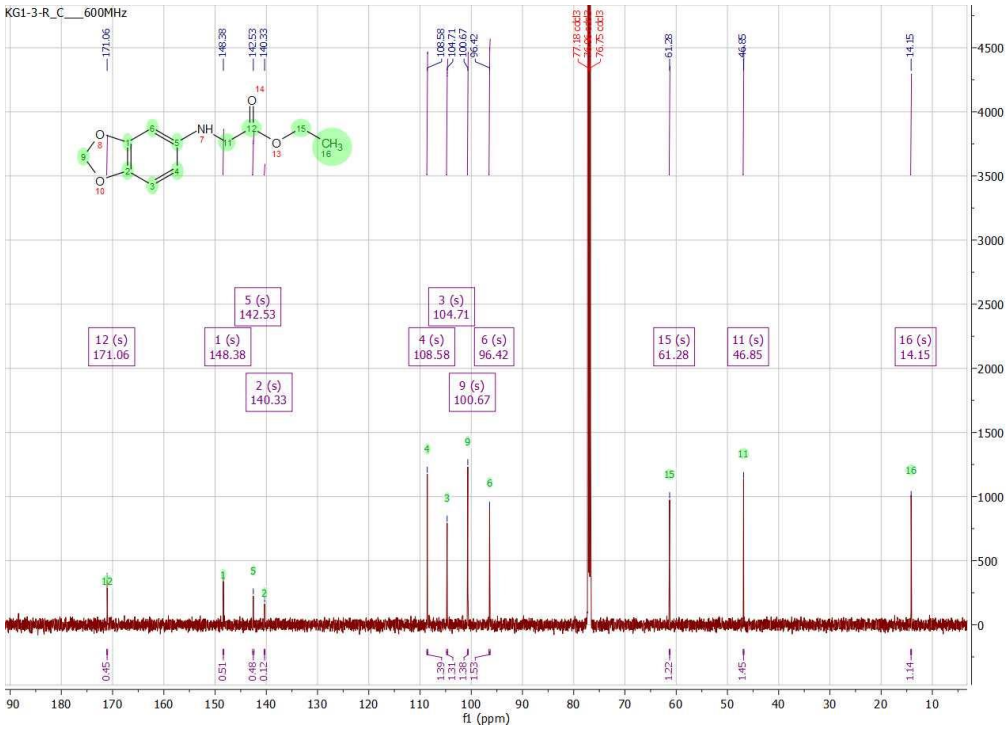
Table of Contents

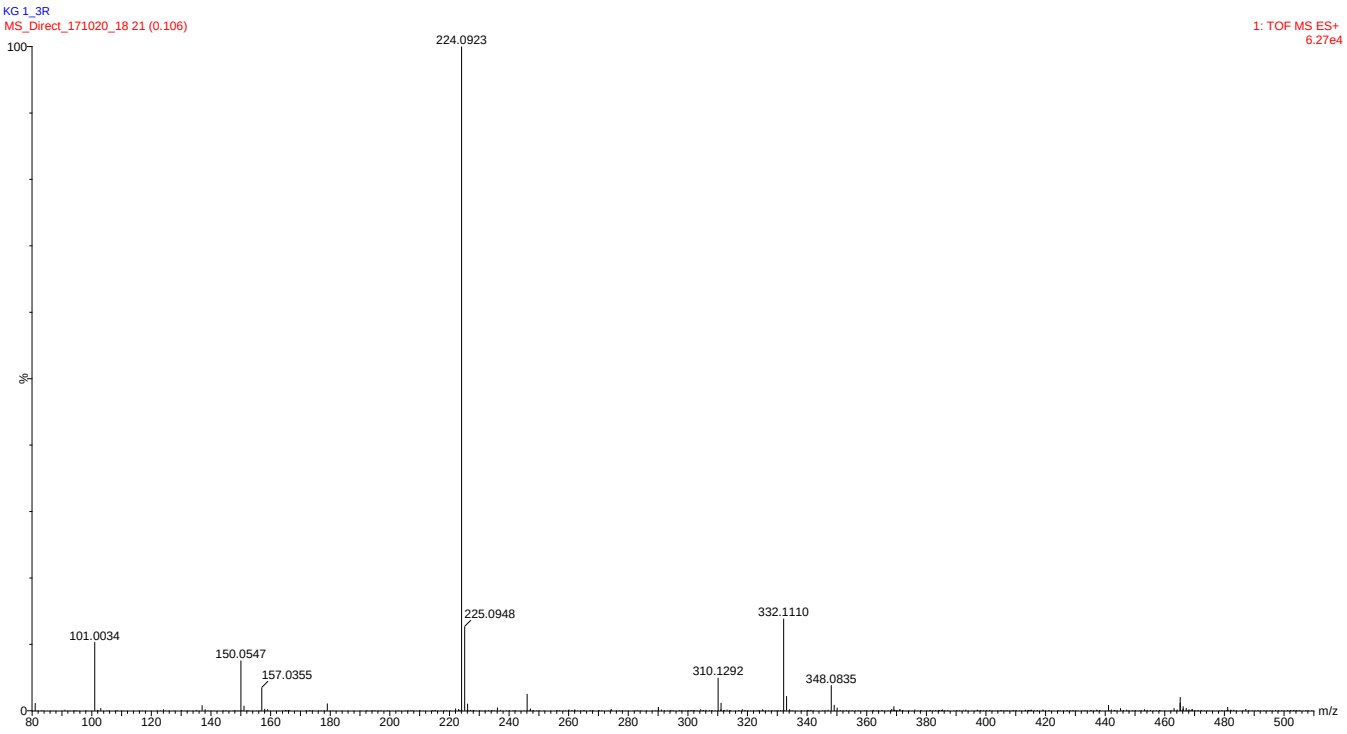
7.1 Library 1

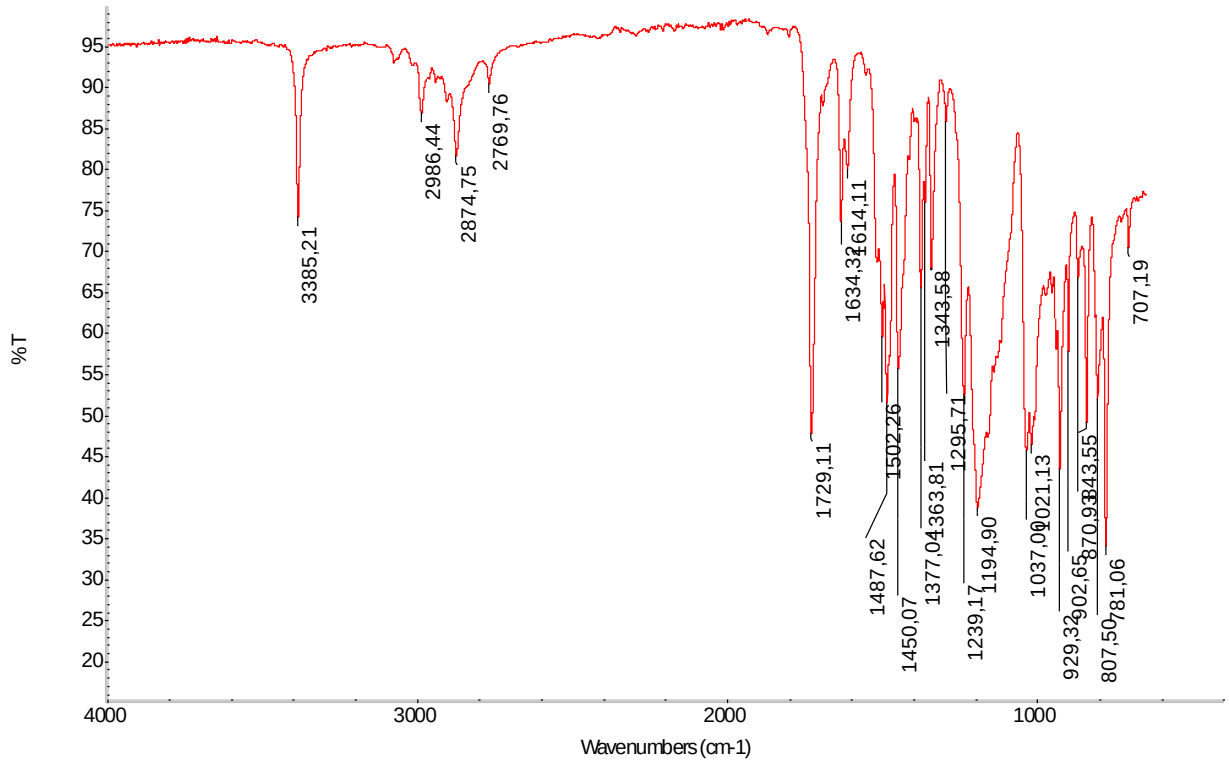
7.1.1 Anilines

7.1.1.1 *Synthesis of Ethyl benzo[d][1,3]dioxol-5-ylglycinate (27)*

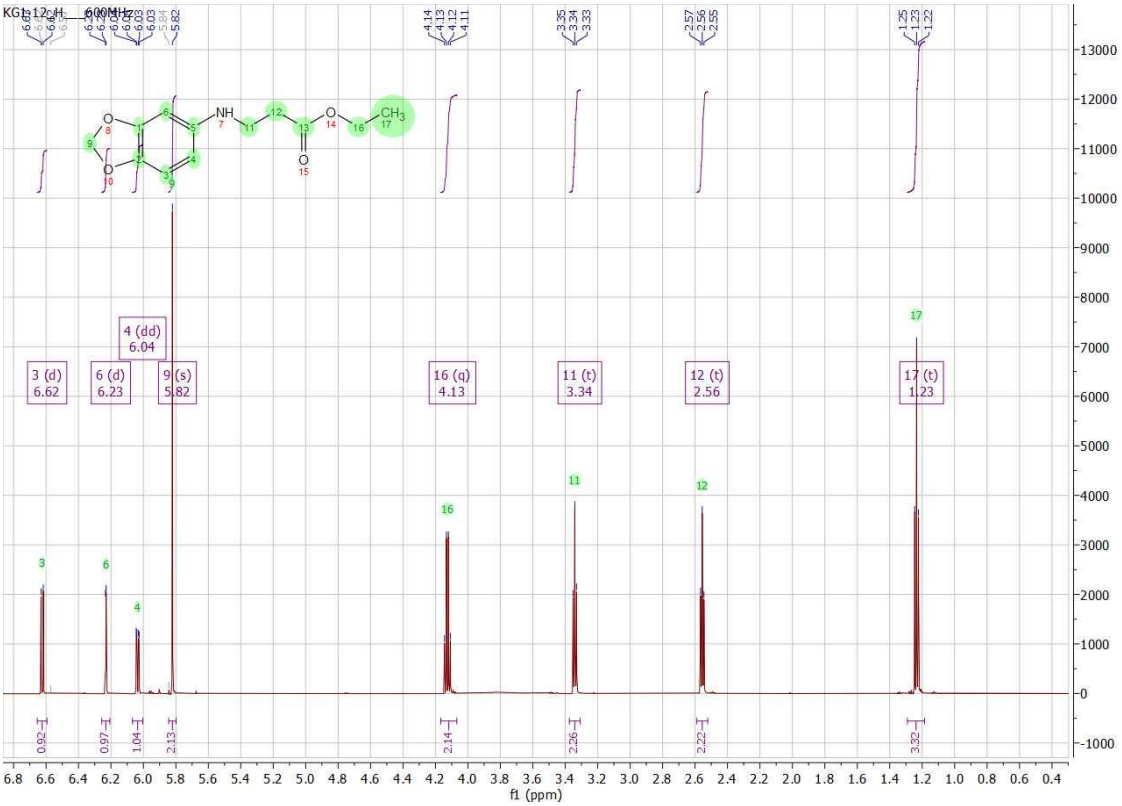


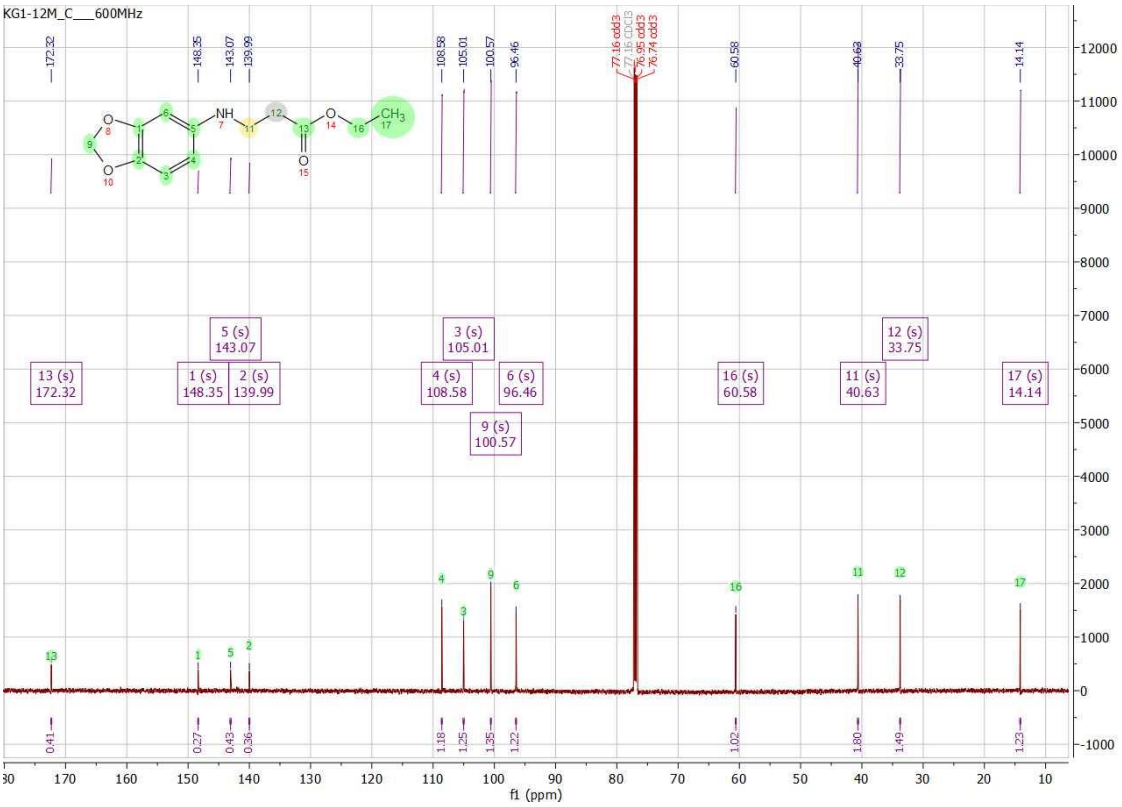


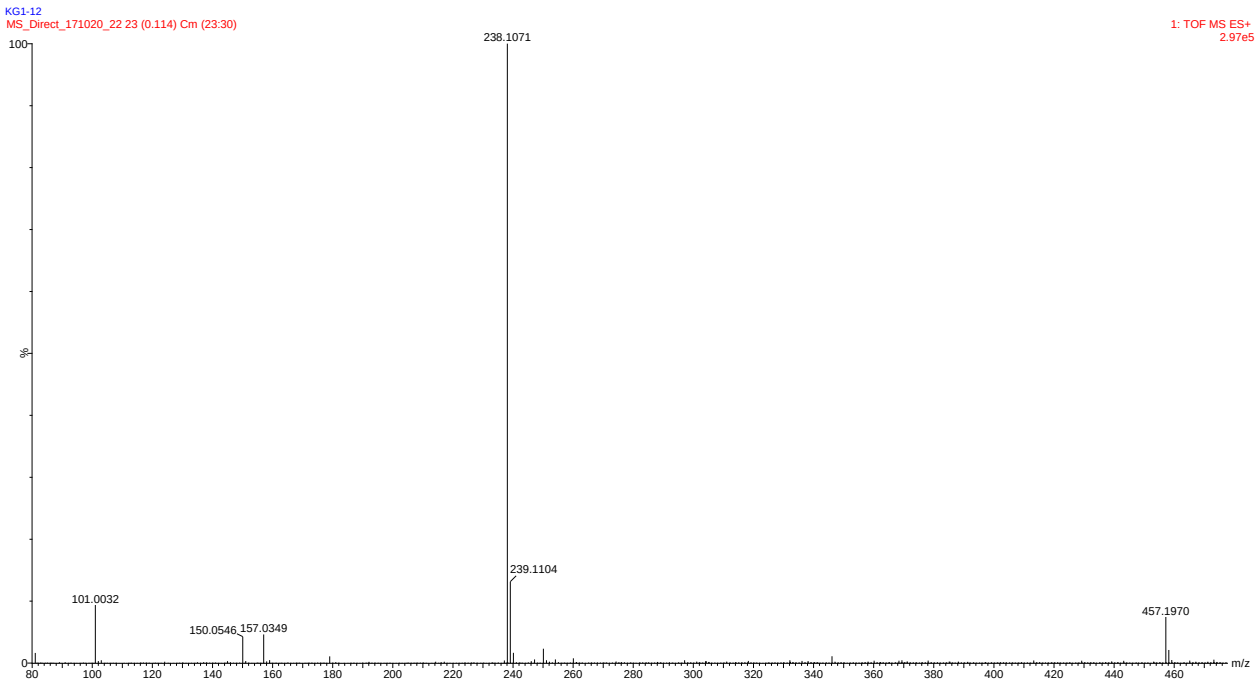




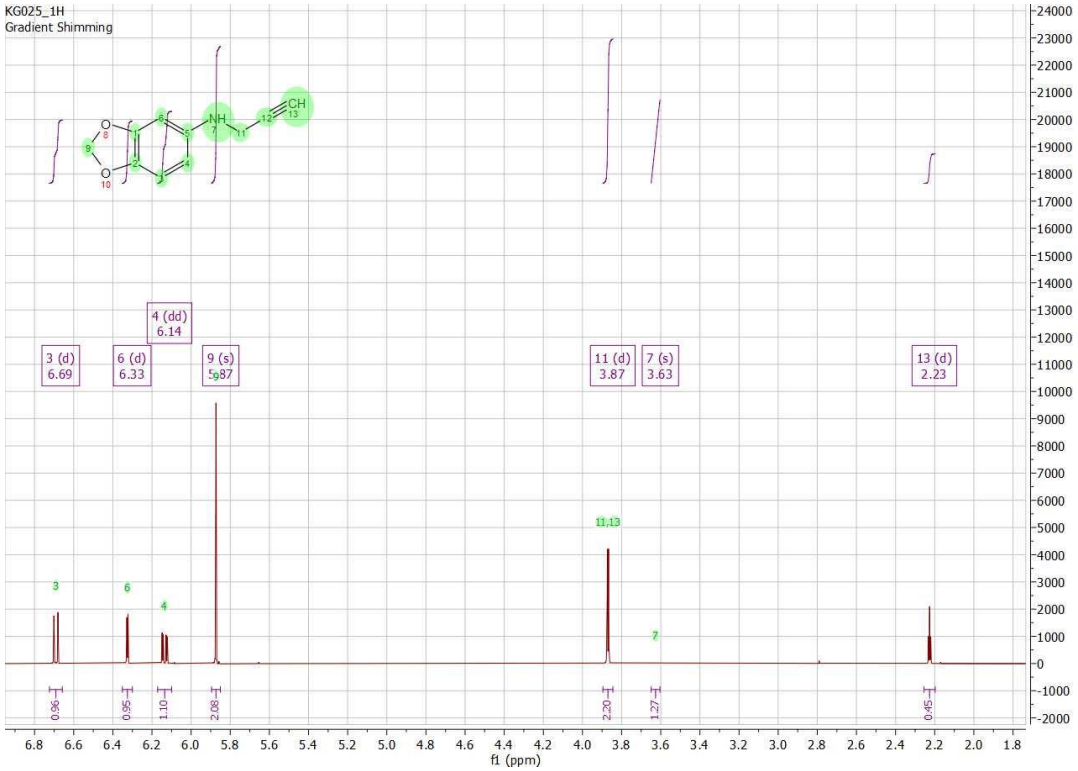
7.1.1.2 *Synthesis of Ethyl 3-(benzo[d][1,3]dioxol-5-ylamino)propanoate* (**28**)

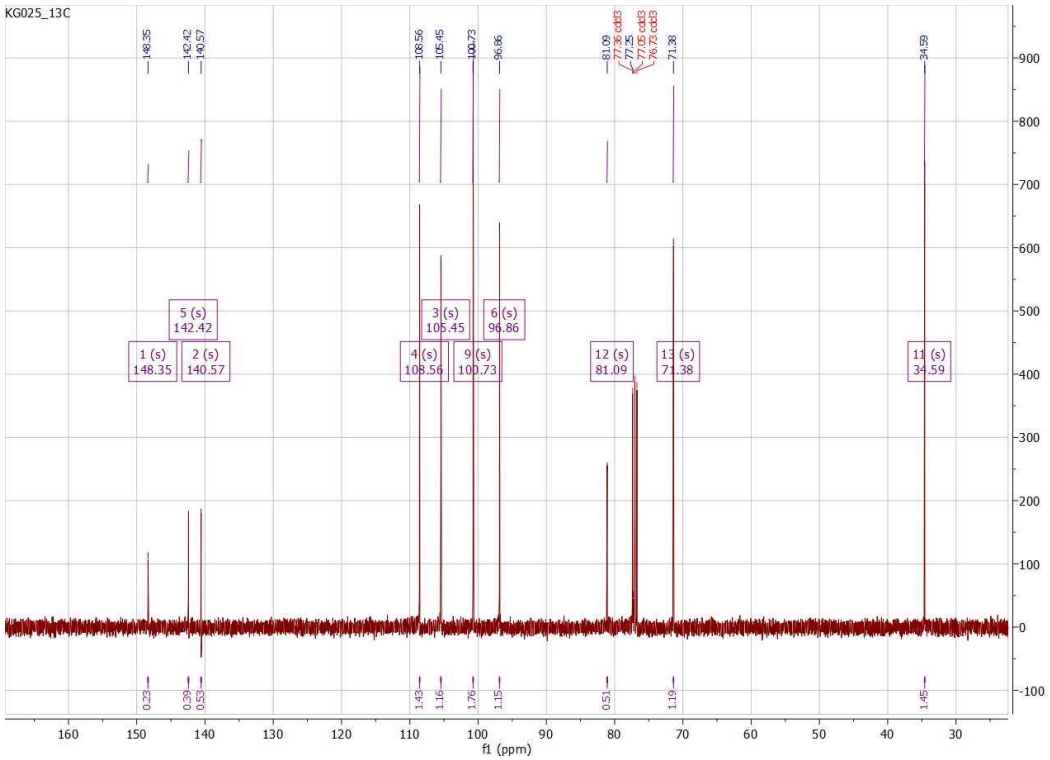


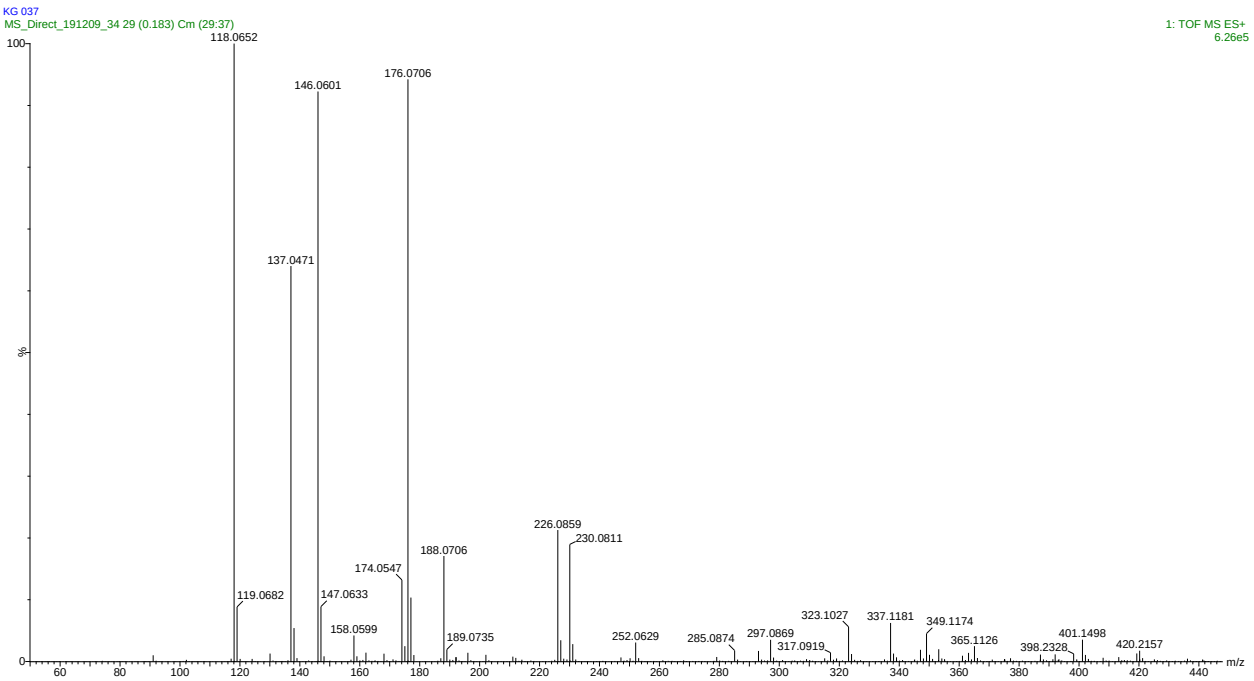


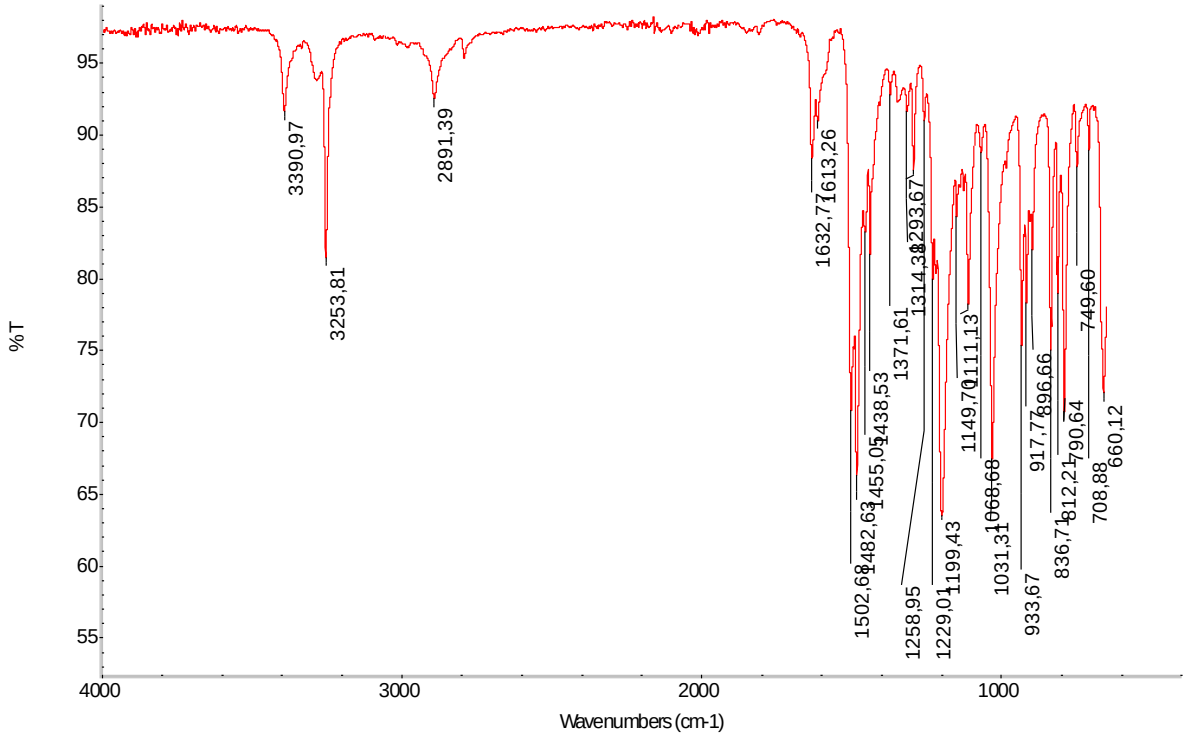


7.1.1.3 *Synthesis of N-(but-3-yn-1-yl)benzo[d][1,3]dioxol-5-amine (29)*

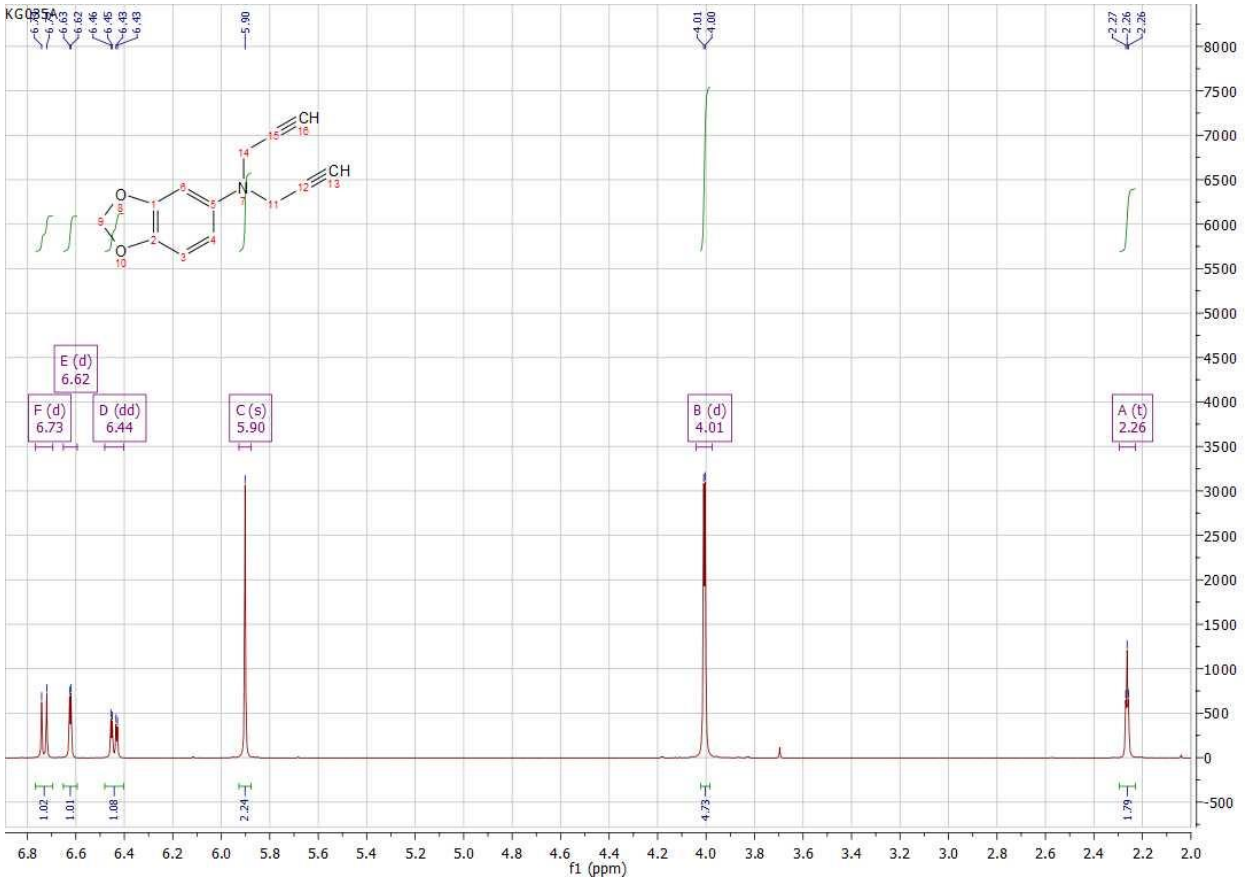




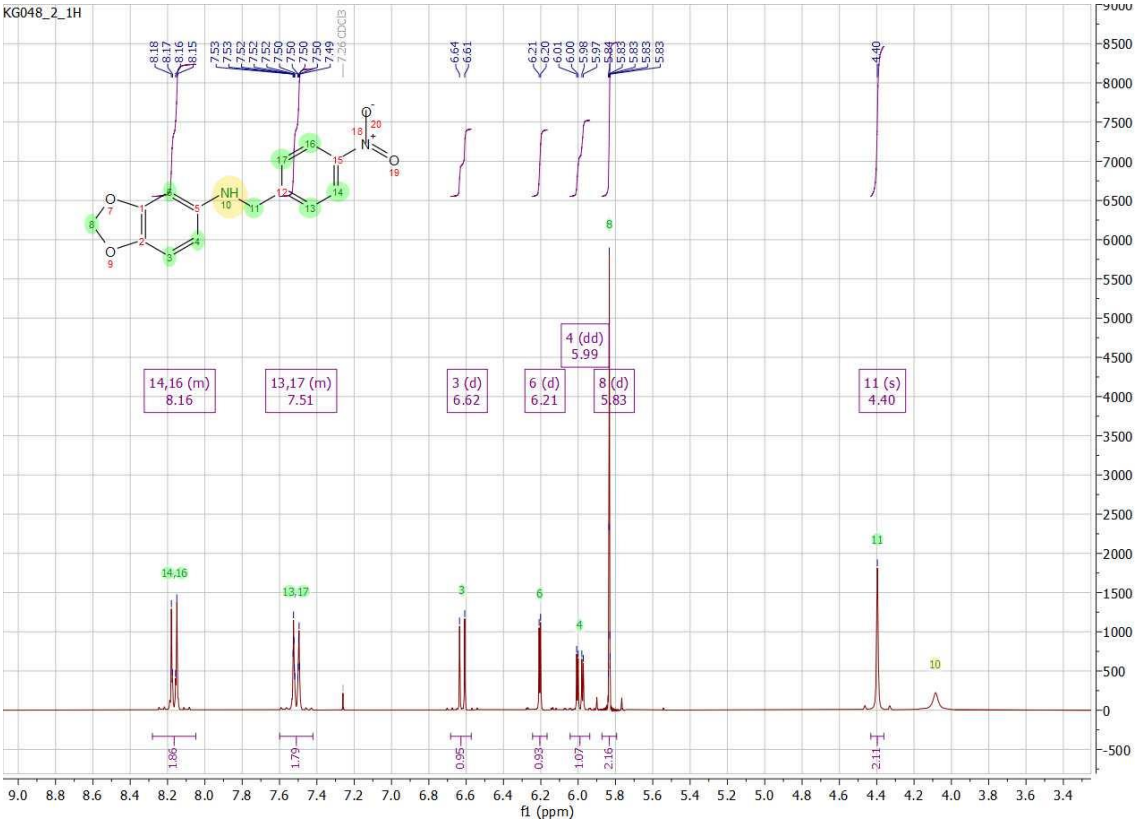


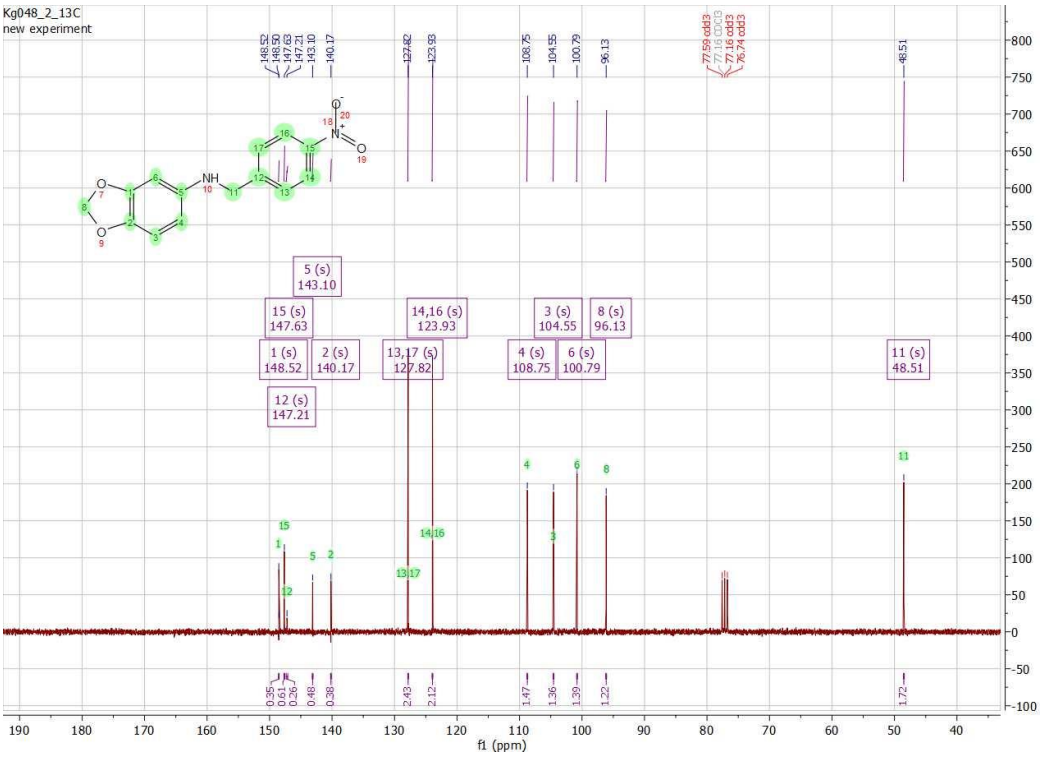


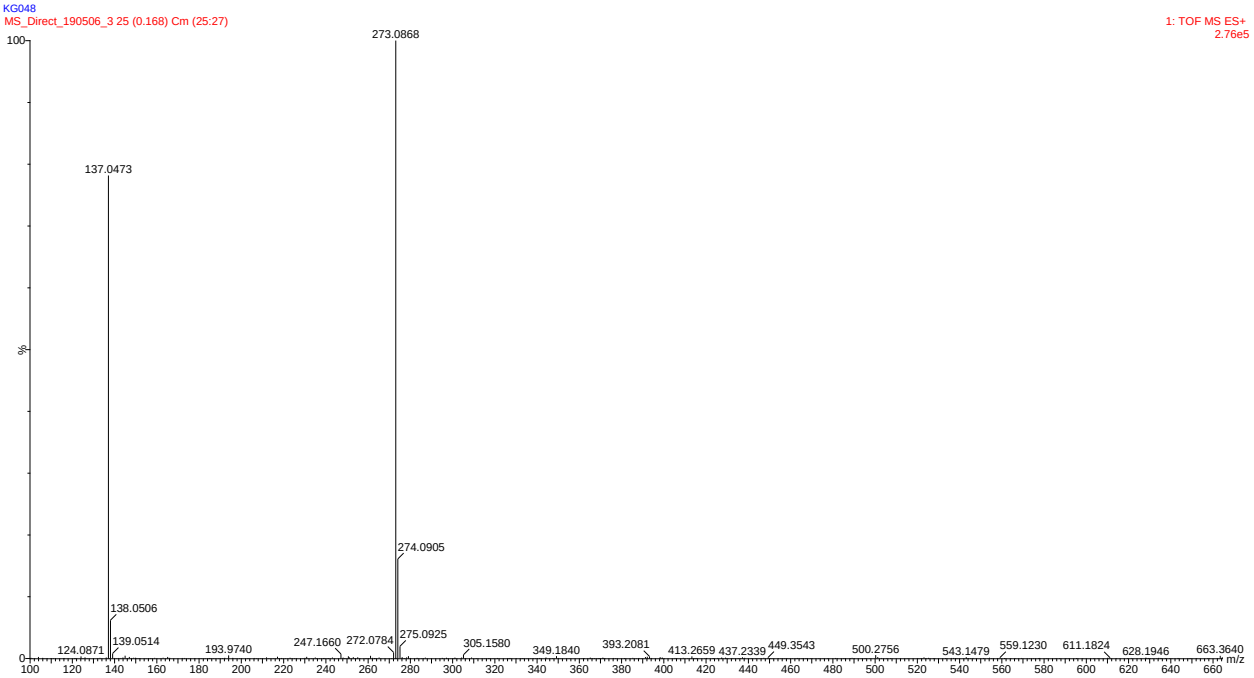
7.1.1.3.1 ^{1}H NMR spectrum of bis-functionalized product

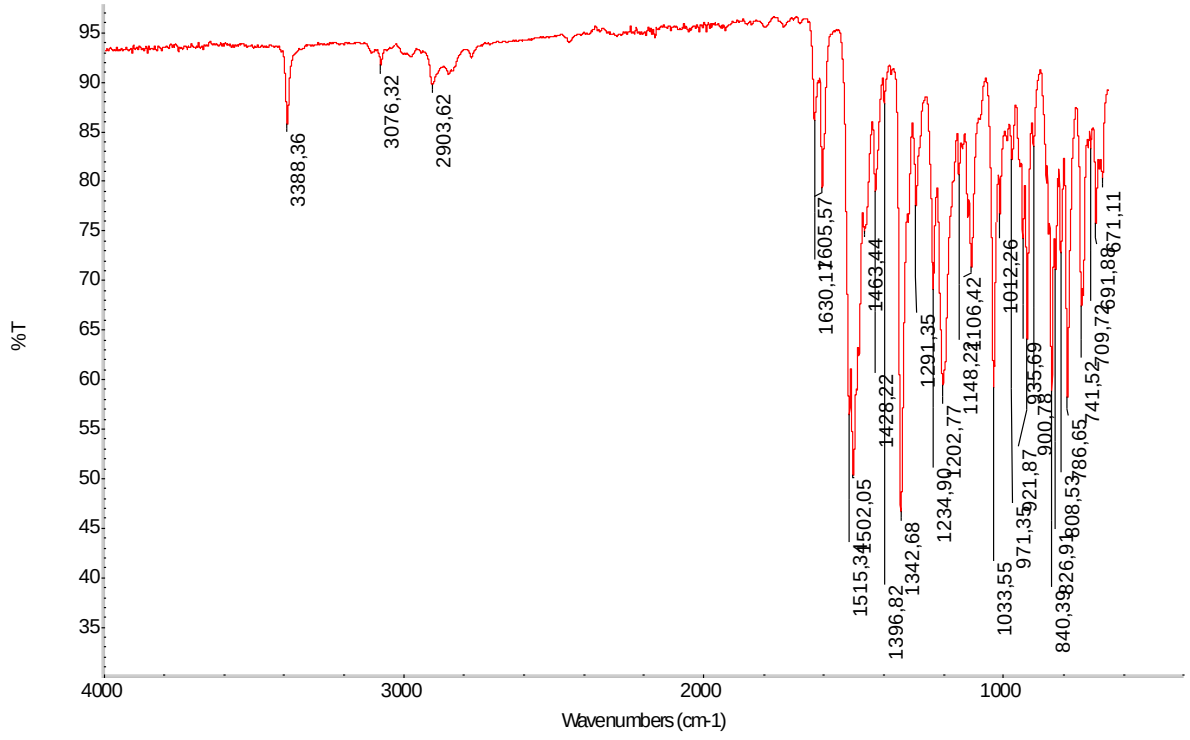


7.1.1.4 *Synthesis of N-(4-nitrobenzyl) benzo[d][1,3]dioxol-5-amine (30)*

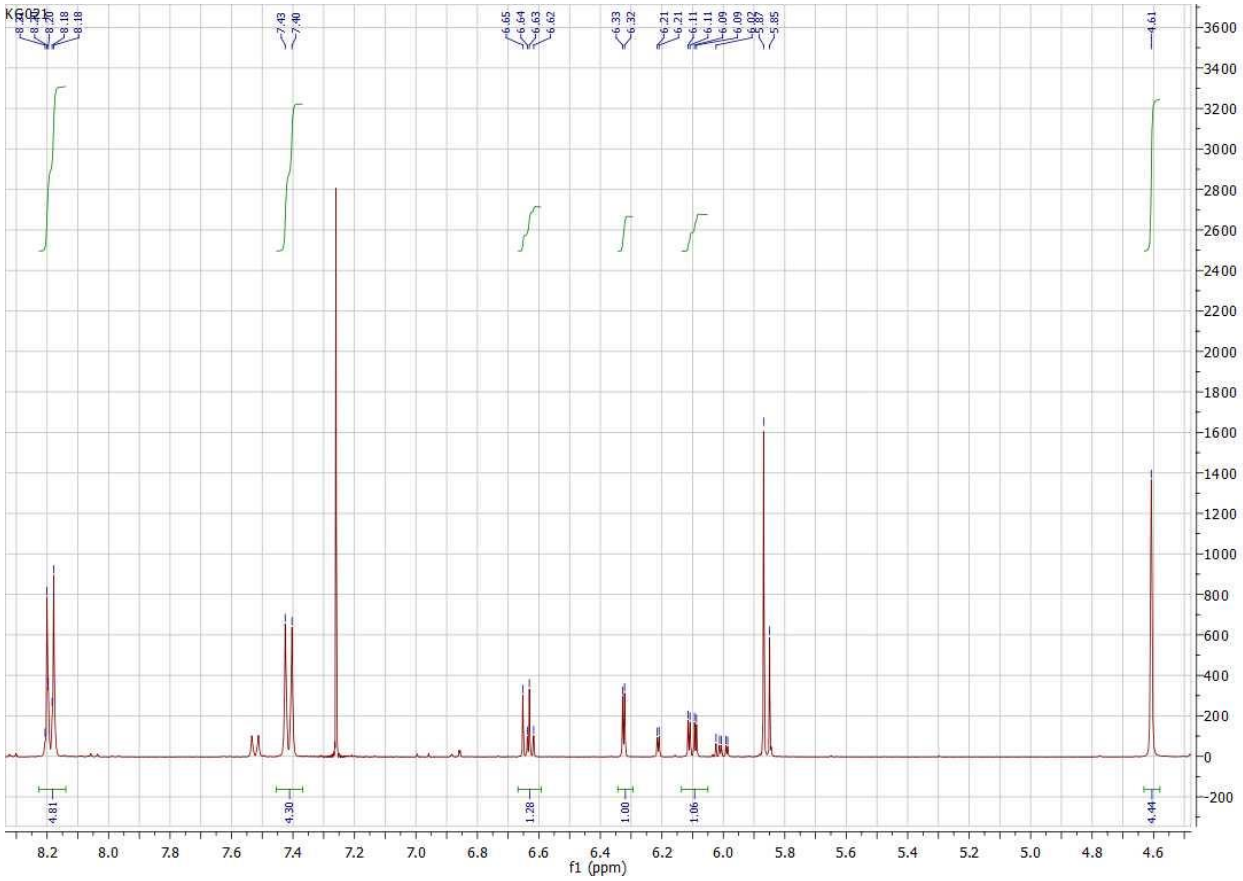




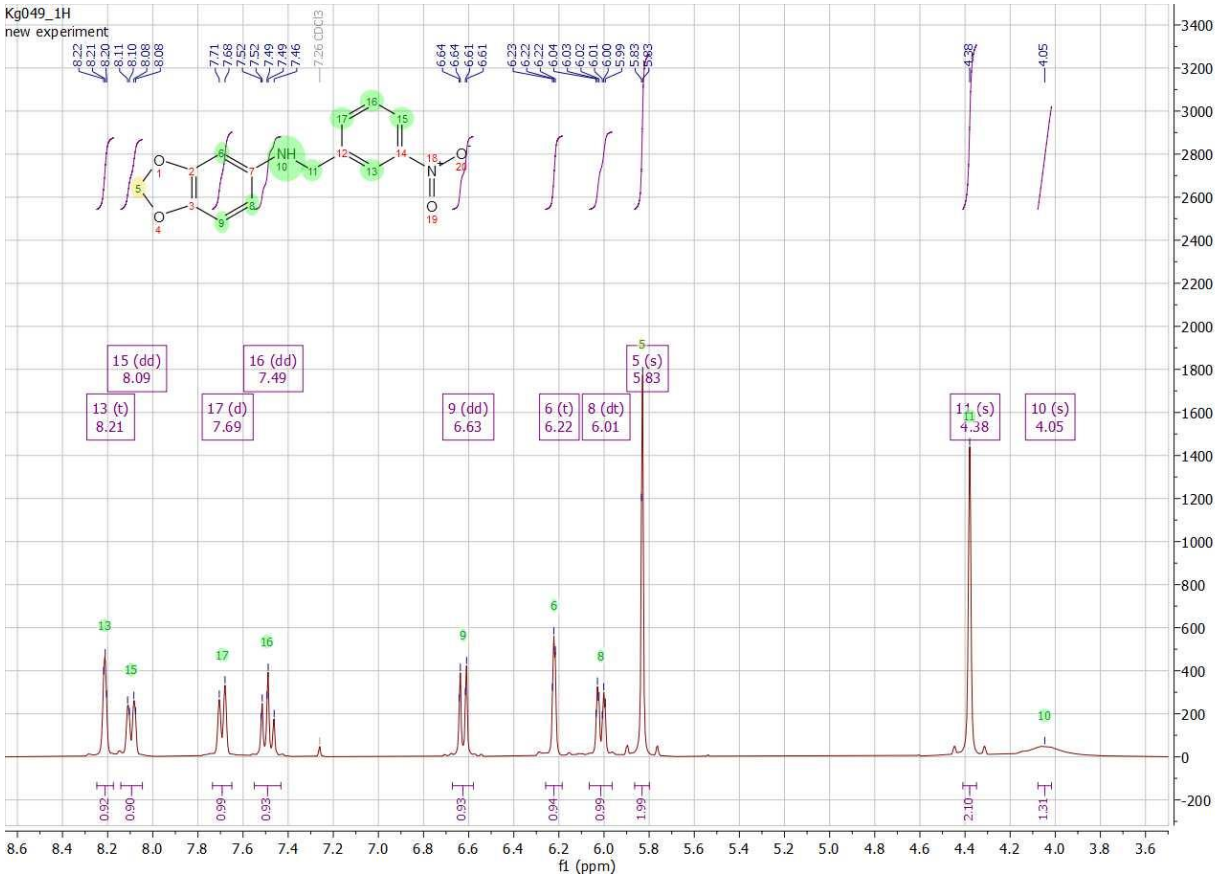


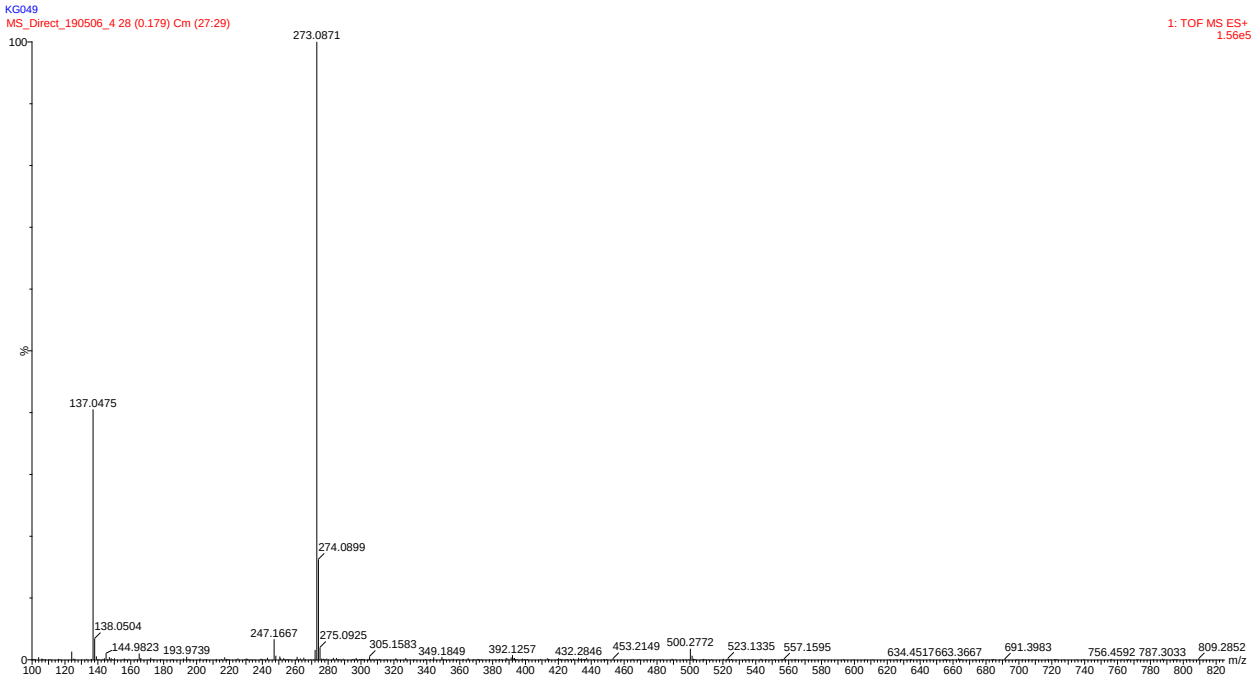


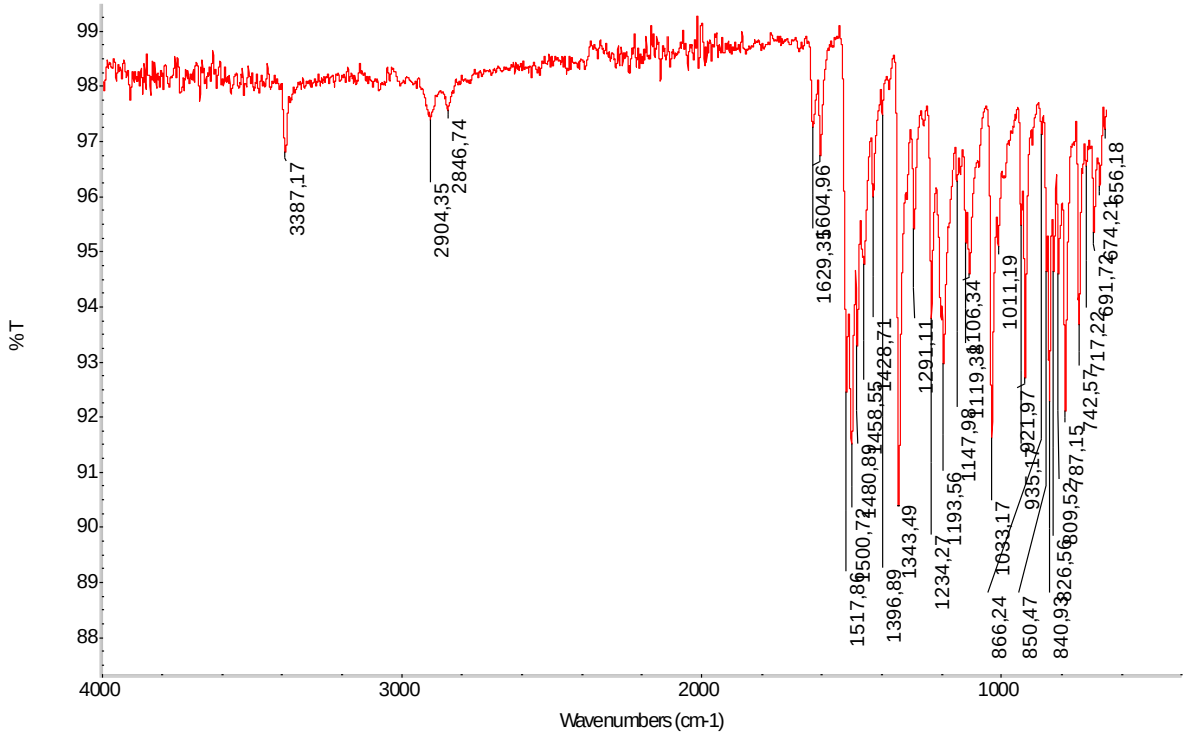
7.1.1.4.1 ^{1}H NMR of bis functionalized product



7.1.1.5 *Synthesis of N-(3-nitrobenzyl)benzo[d][1,3]dioxol-5-amine (31)*

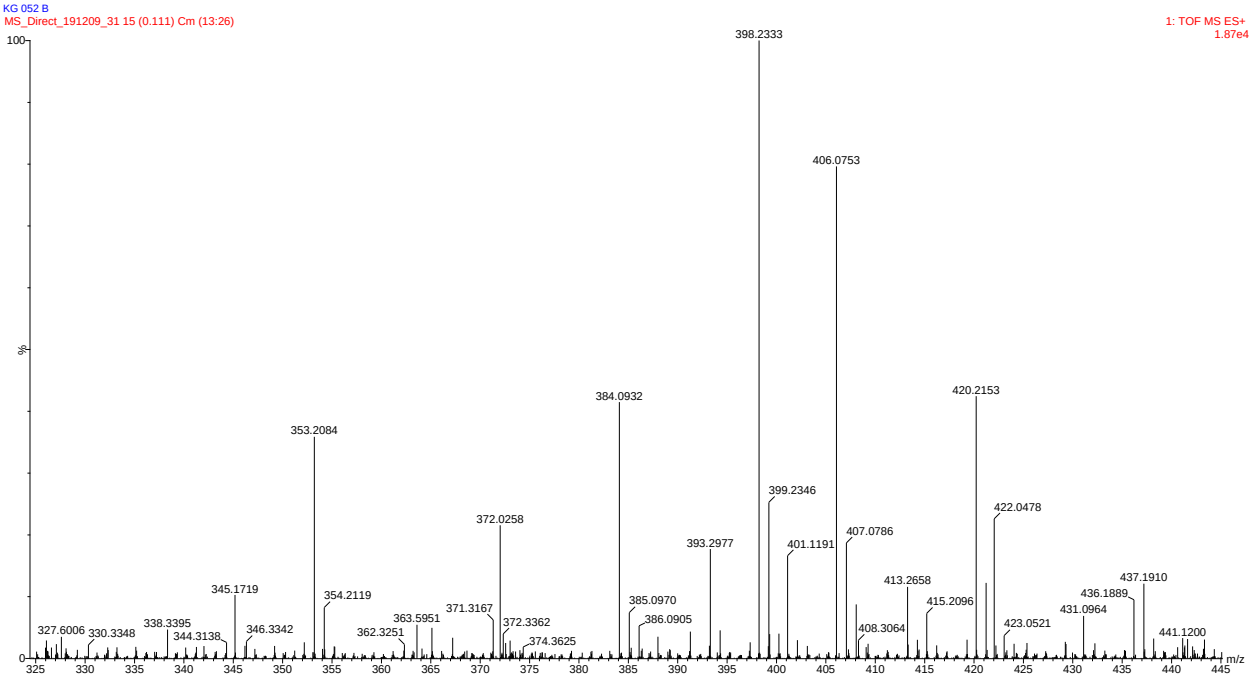




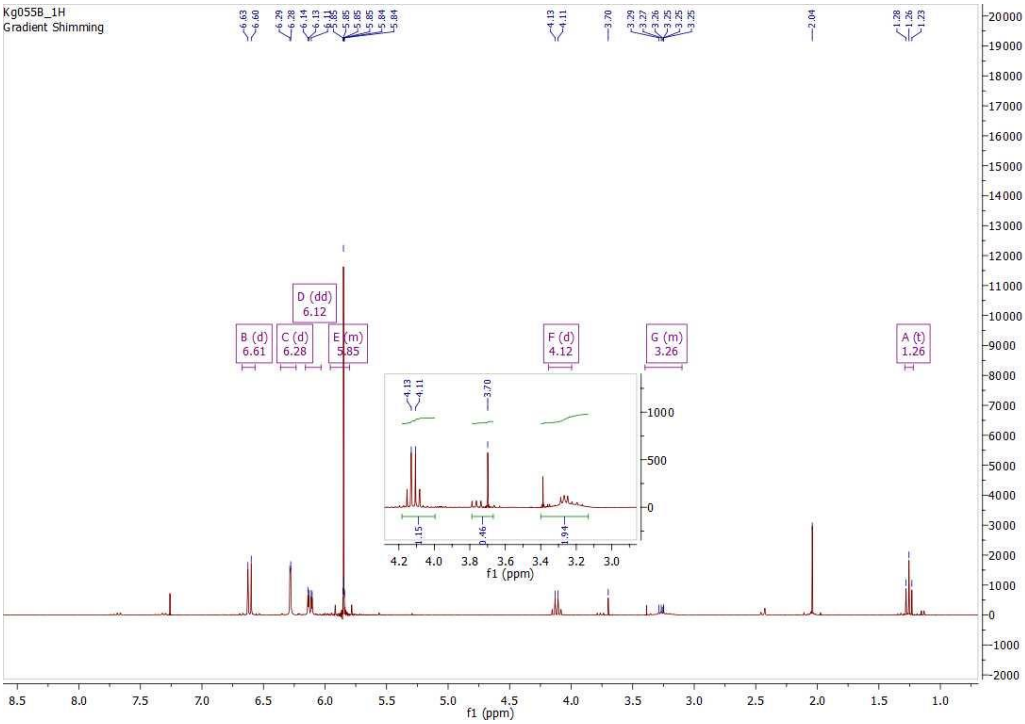


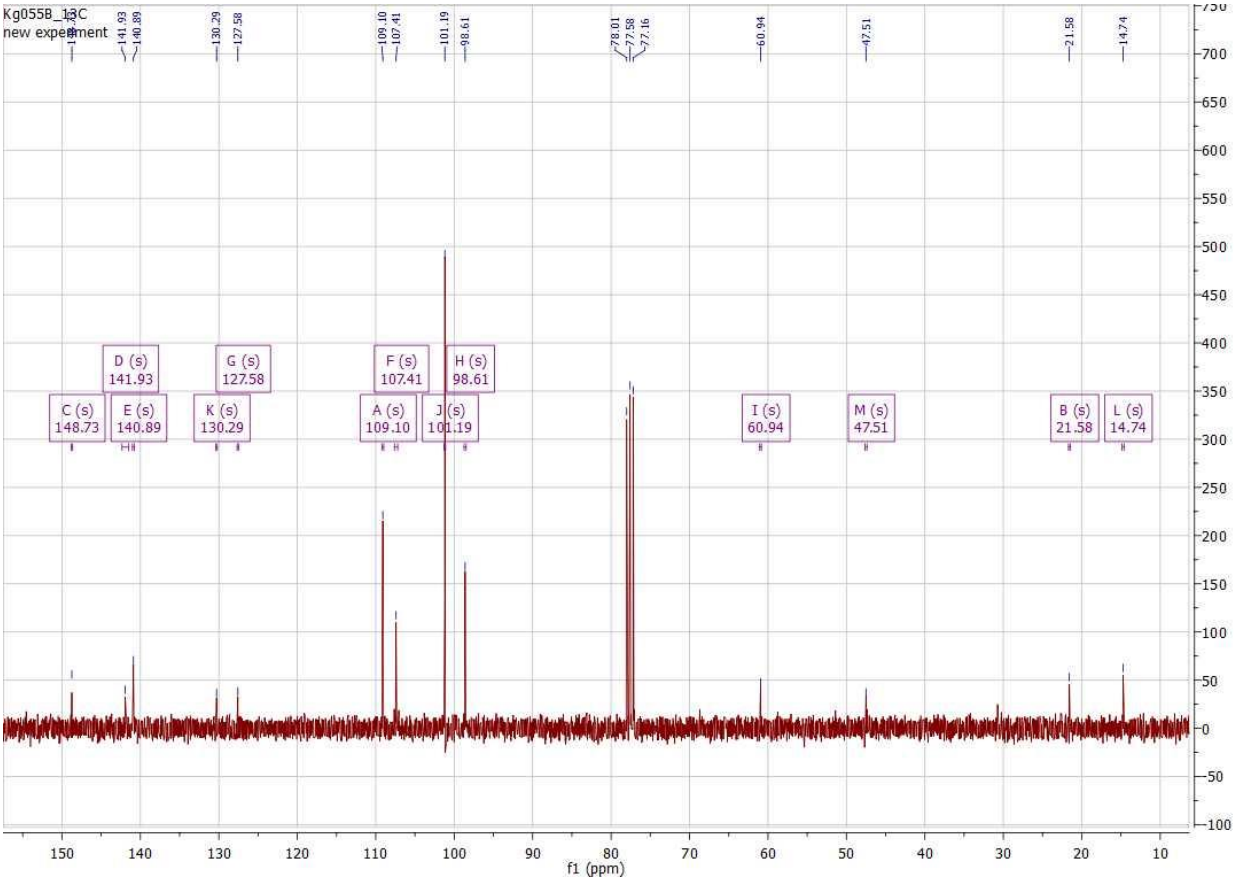
7.1.1.6 *Synthesis of N-(3-(benzo[d][1,3]dioxol-5-ylamino)propyl)-4-methylbenzenesulfonamide (32)*

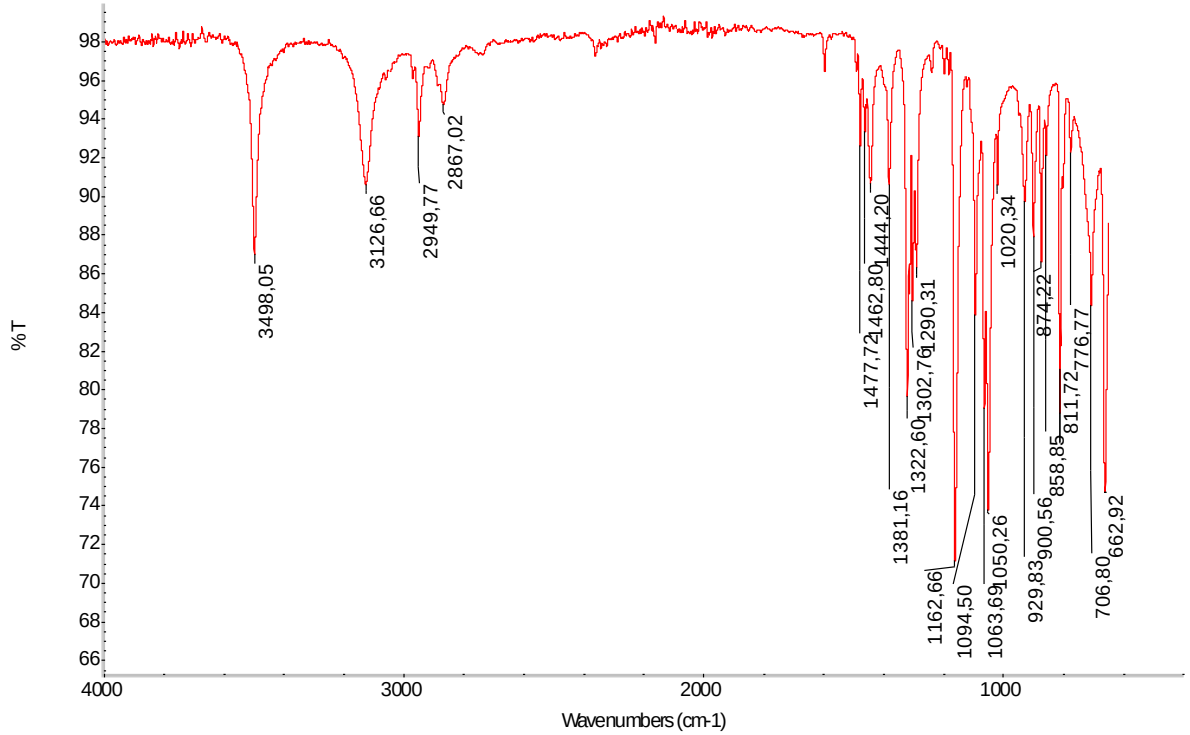
7.1.1.6.1 Mass spectrum of synthesized starting material



*7.1.1.6.2 Product **32** spectrum, showing the presence of the final product as well as the starting material **SM1**.*

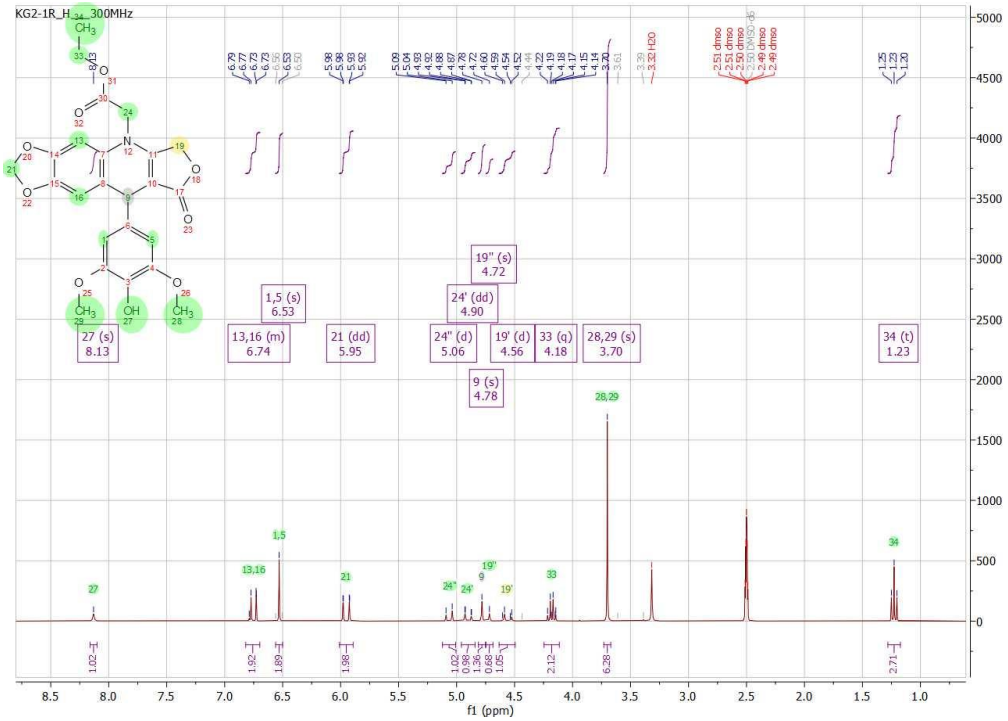


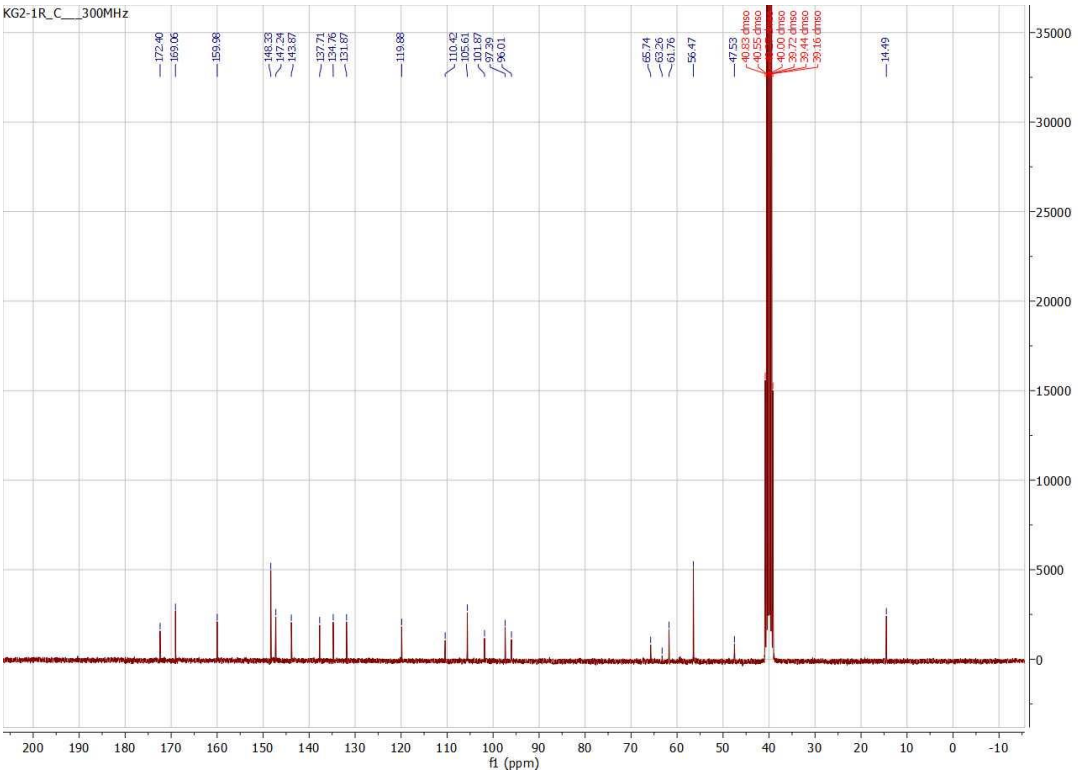


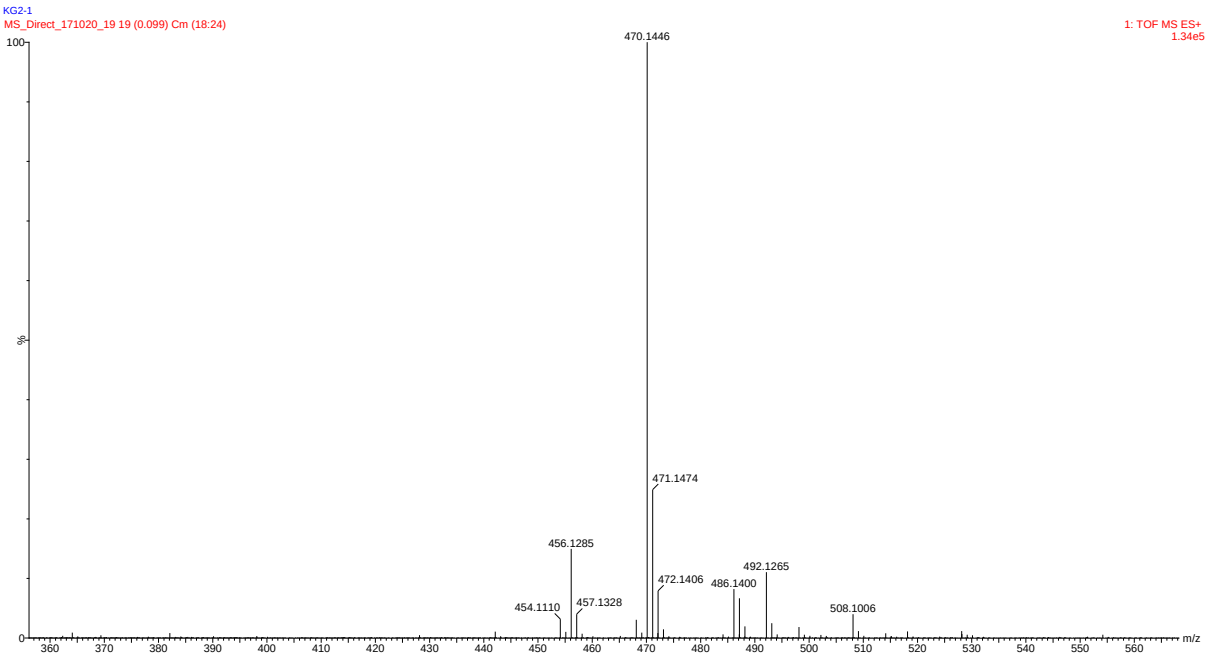


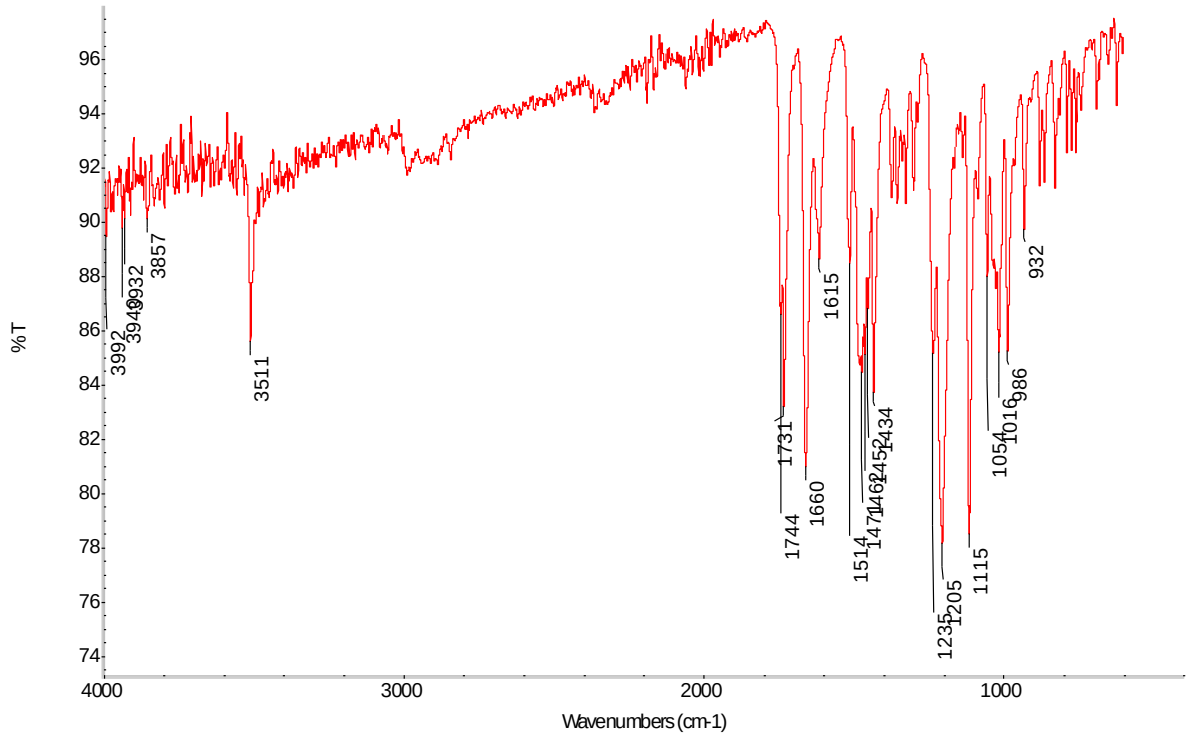
7.1.2 **4-aza-podophyllotoxin analogues**

7.1.2.1 ***Analysis of ethyl 2-[9-(4-hydroxy-3,5-dimethoxyphenyl)-8-oxo-6,9-dihydro-[1,3]dioxolo[4,5-g] furo[3,4-b] quinolin-5(8H)-yl]acetate (33a)***

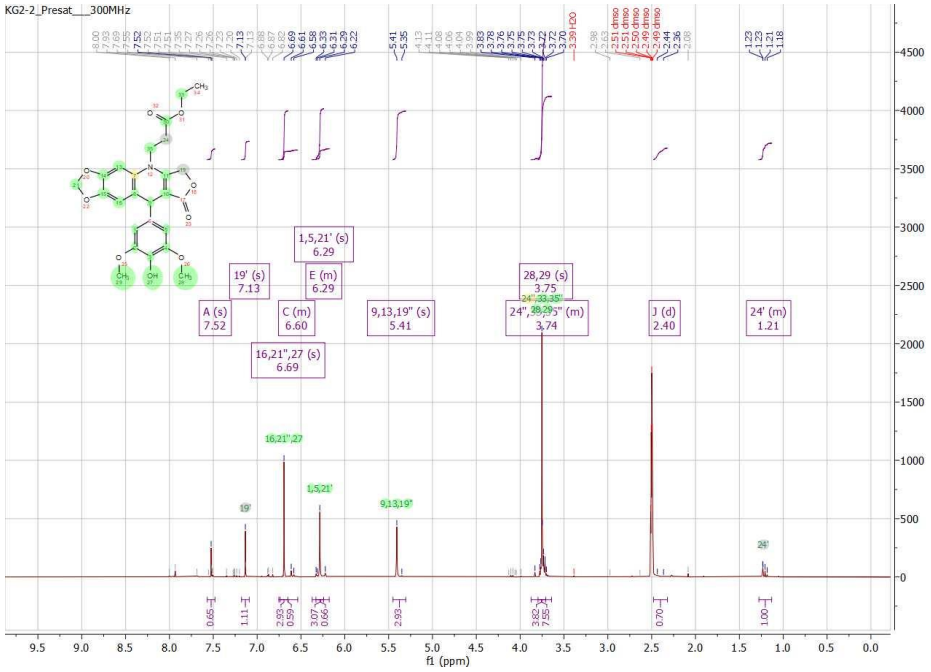


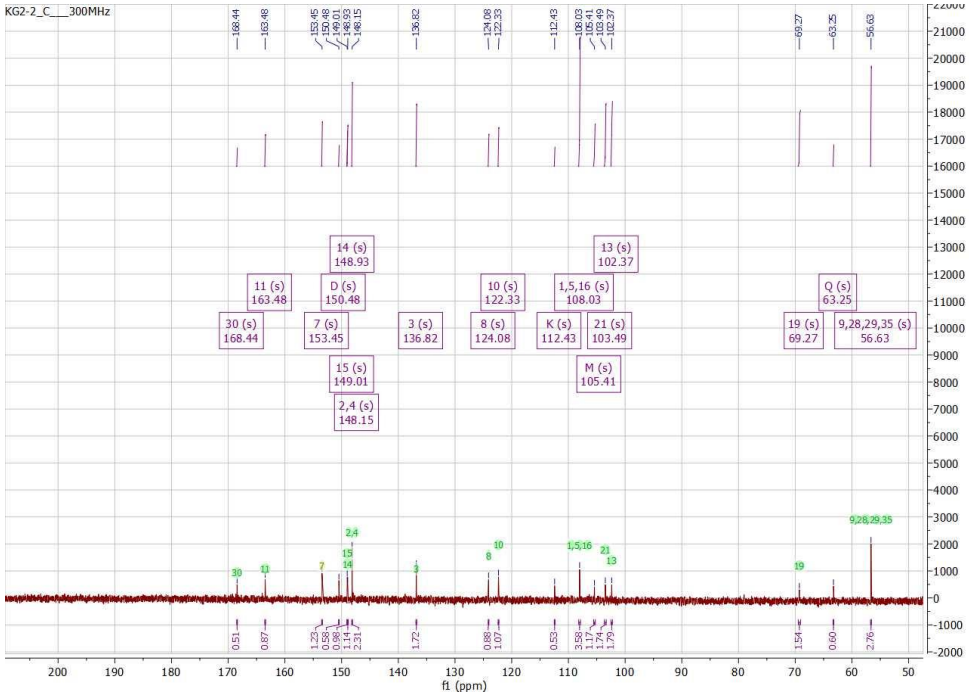


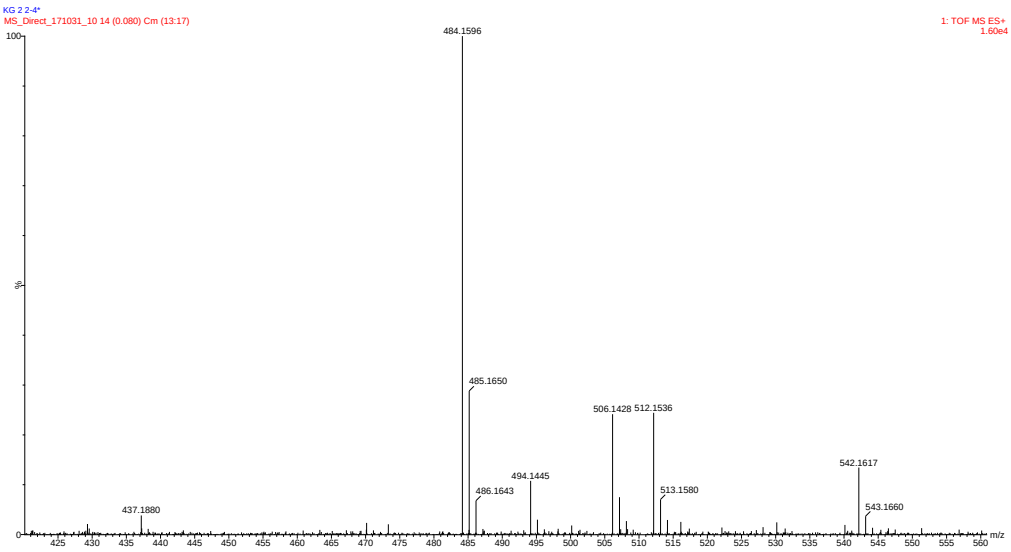


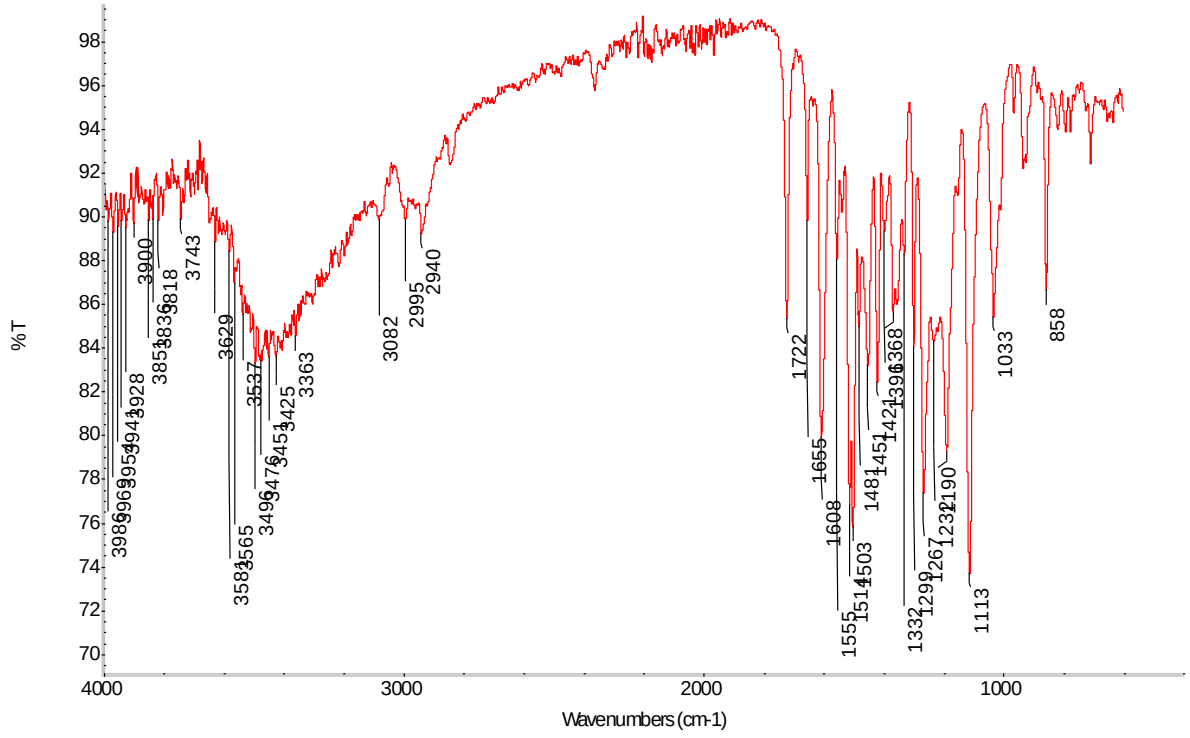


7.1.2.2 *Analysis of ethyl 3-(9-(4-hydroxy-3,5-dimethoxyphenyl)-8-oxo-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-5(6H,8H,9H)-yl)propanoate (34a)*

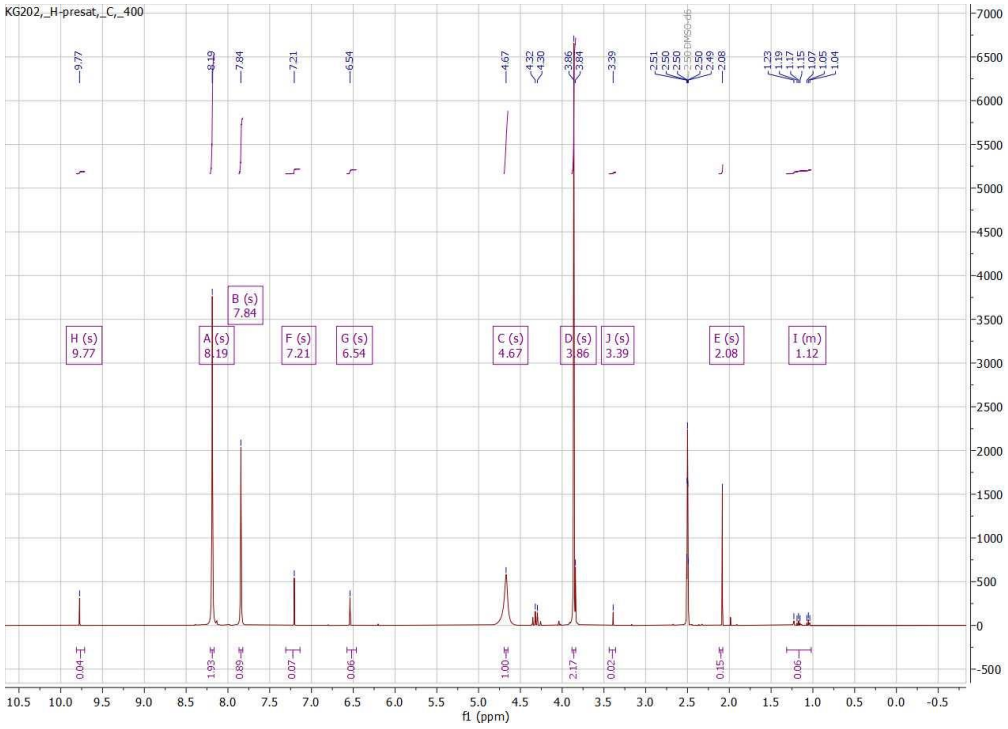


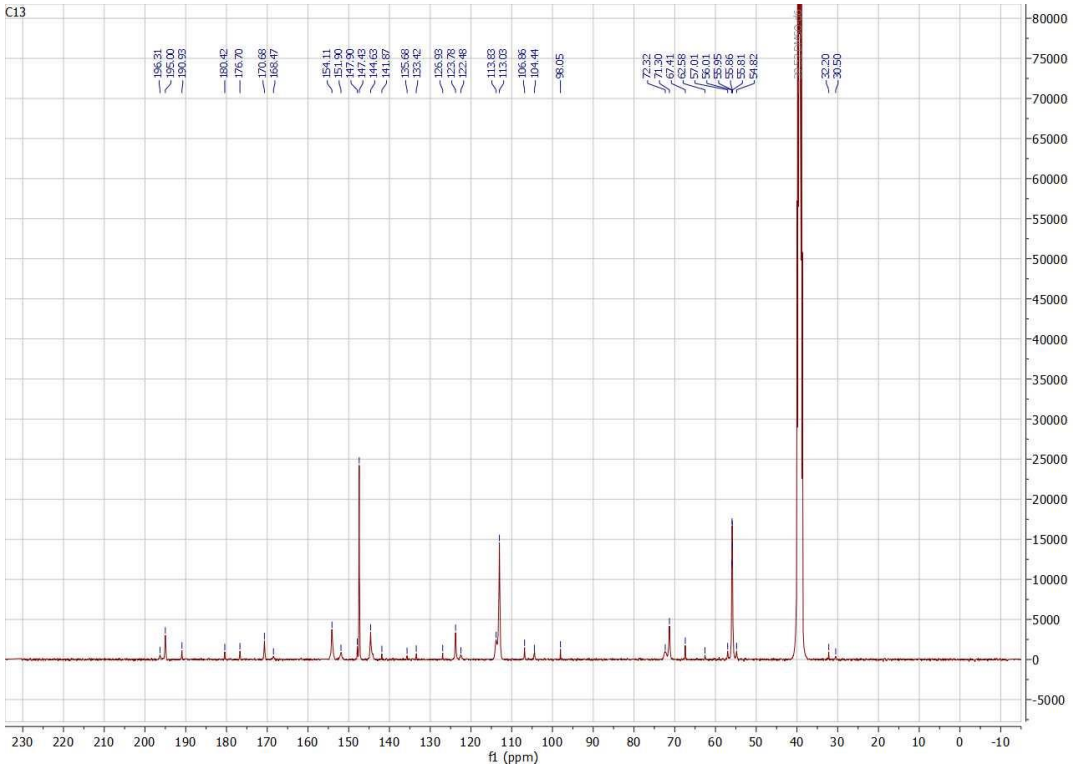


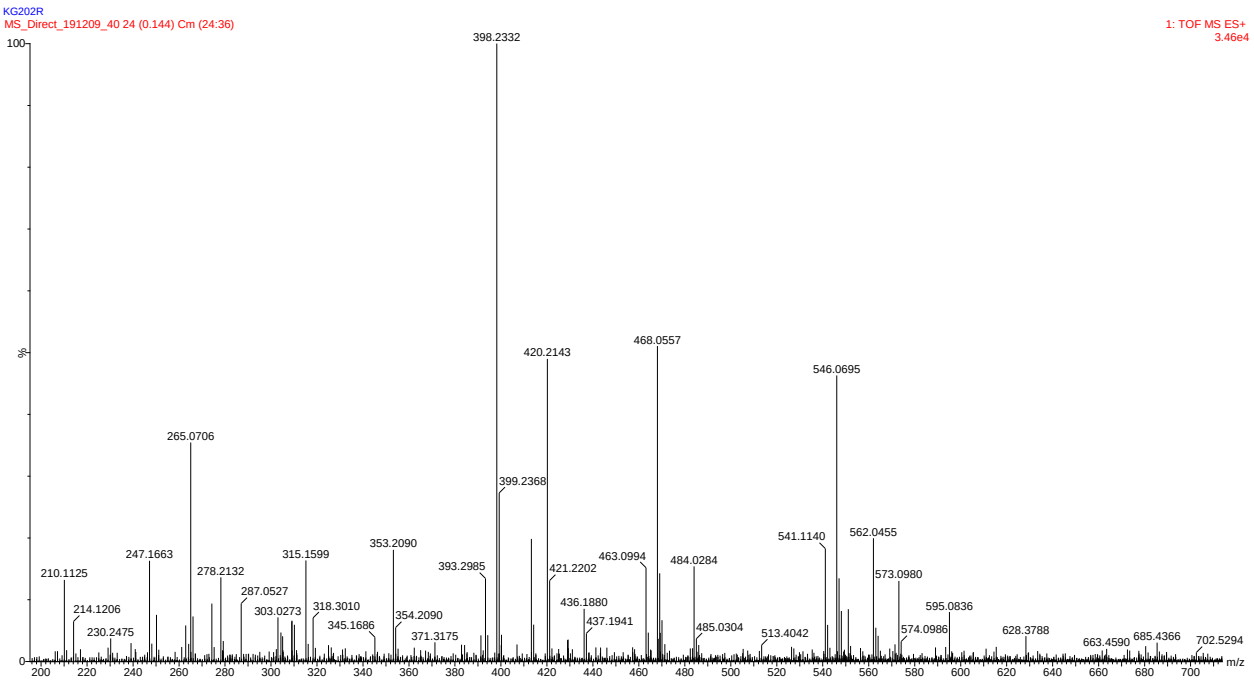


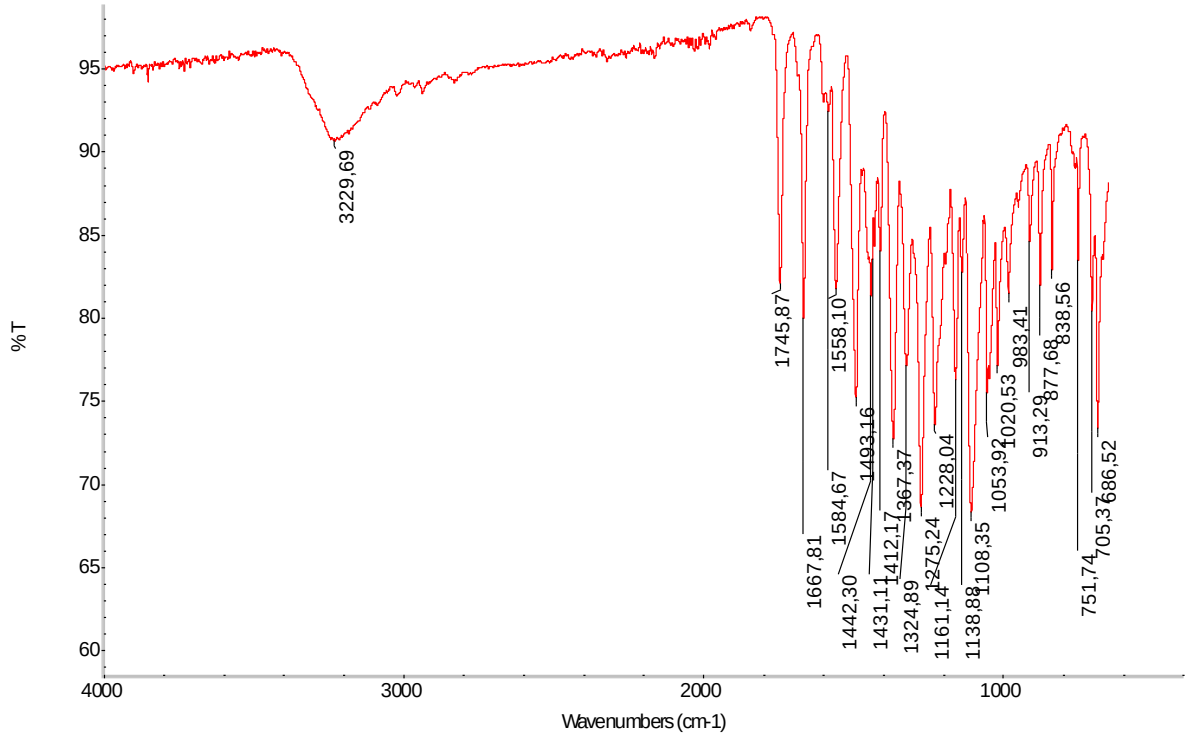


7.1.2.3 *Analysis for unknown compound initially thought to be 35a*

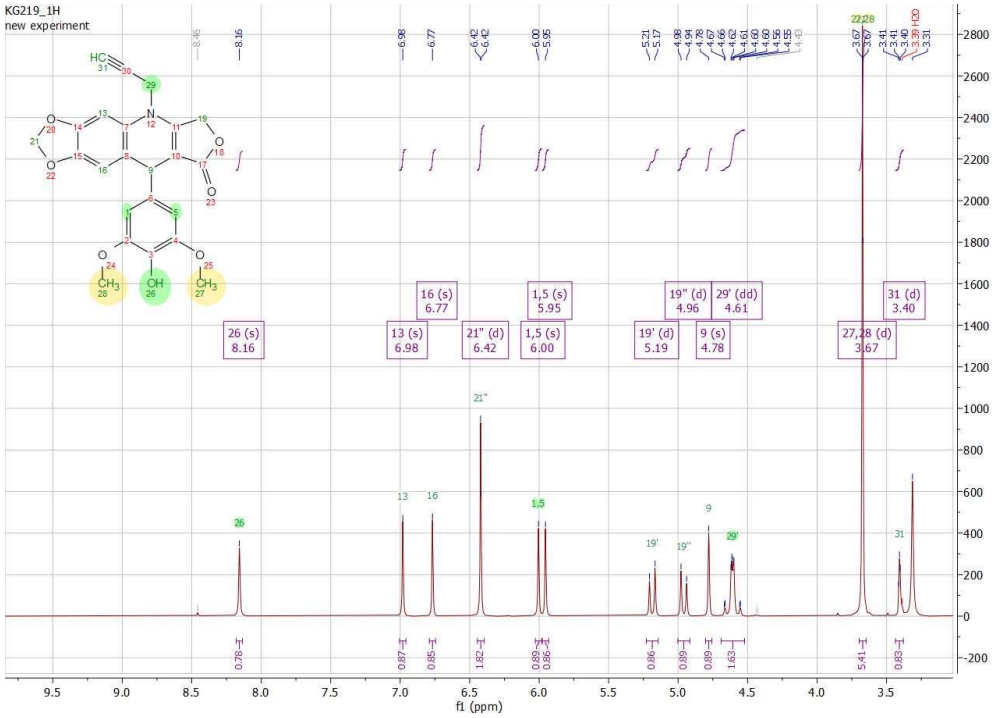


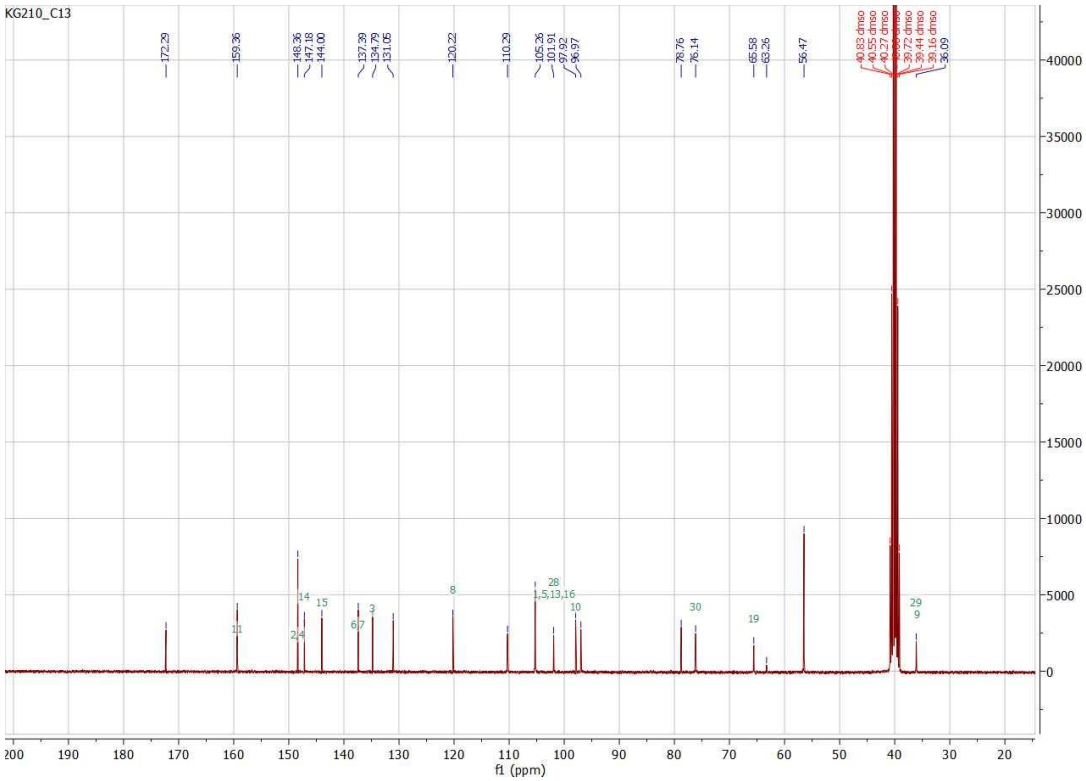


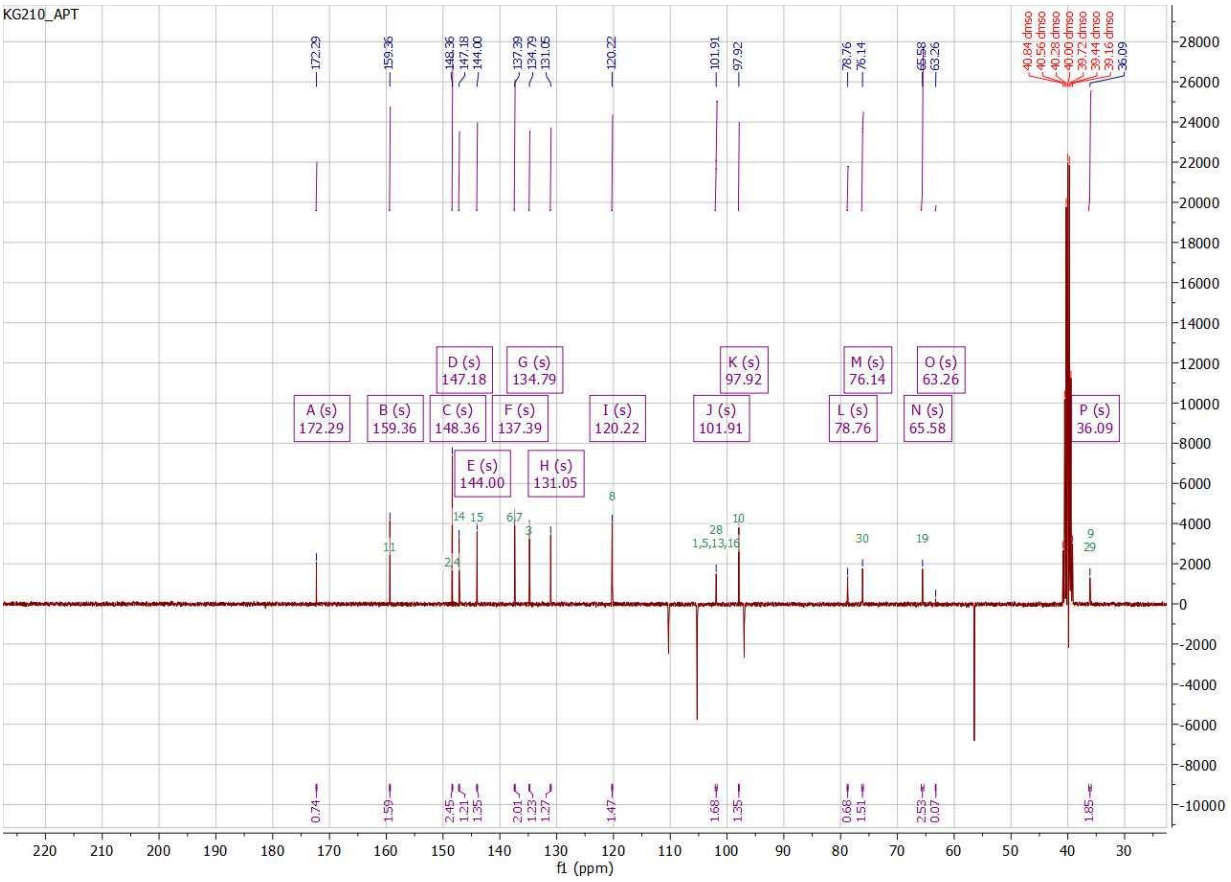


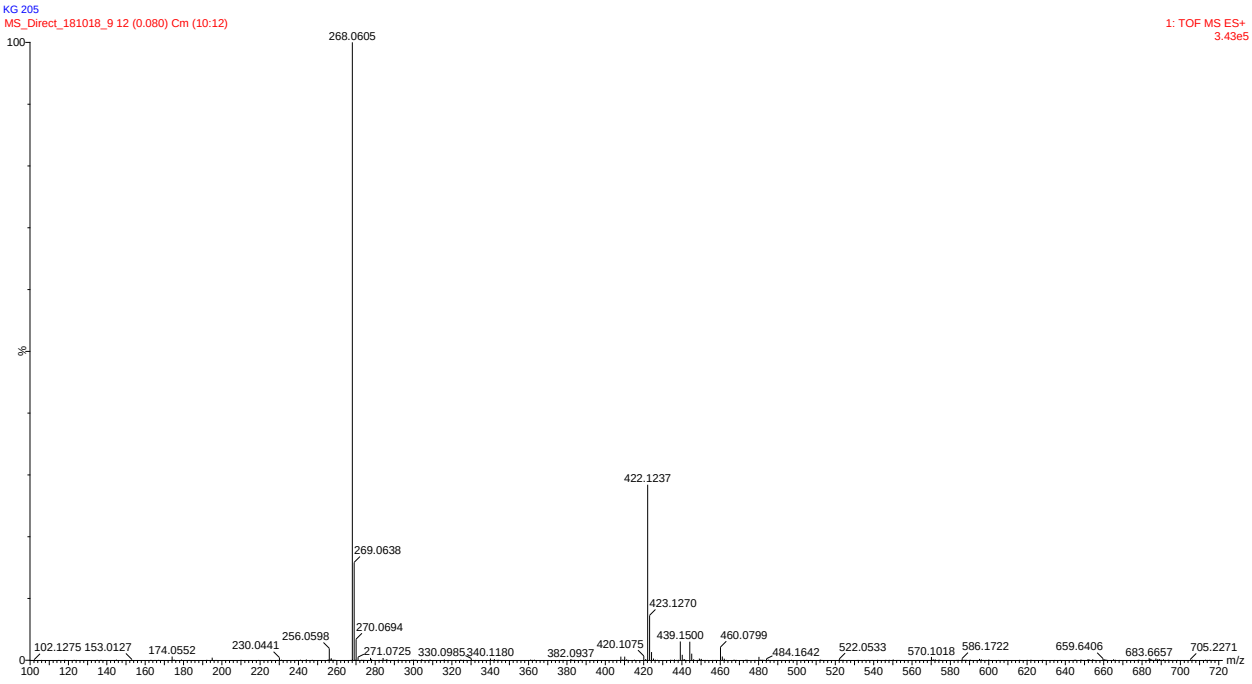


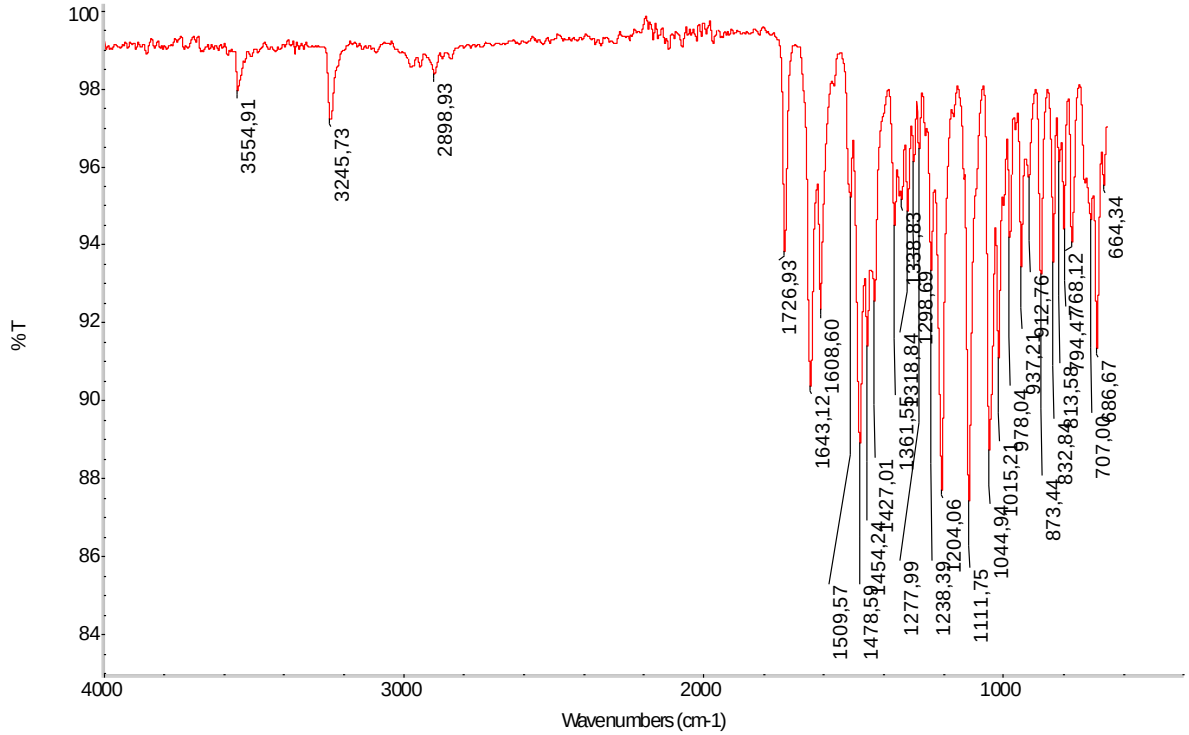
7.1.2.4 *Analysis of 9-(4-hydroxy-3,5-dimethoxyphenyl)-5-(prop-2-yn-1-yl)-6,9-dihydro-[1,3]dioxolo [4,5-g]furo[3,4-b]quinolin-8(5H)-one (36a)*



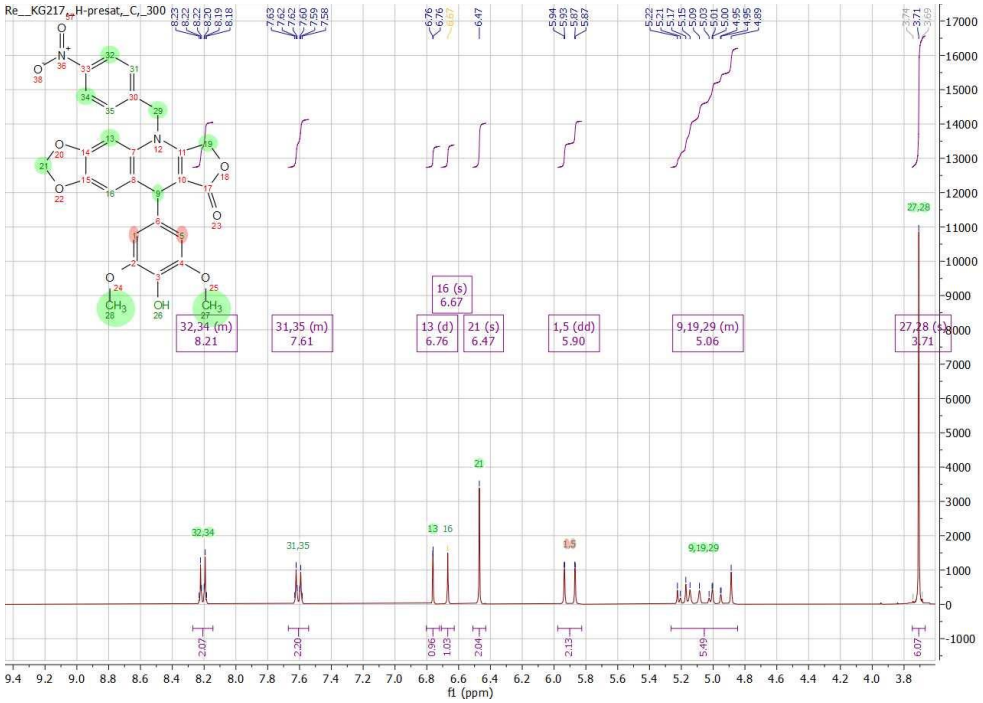


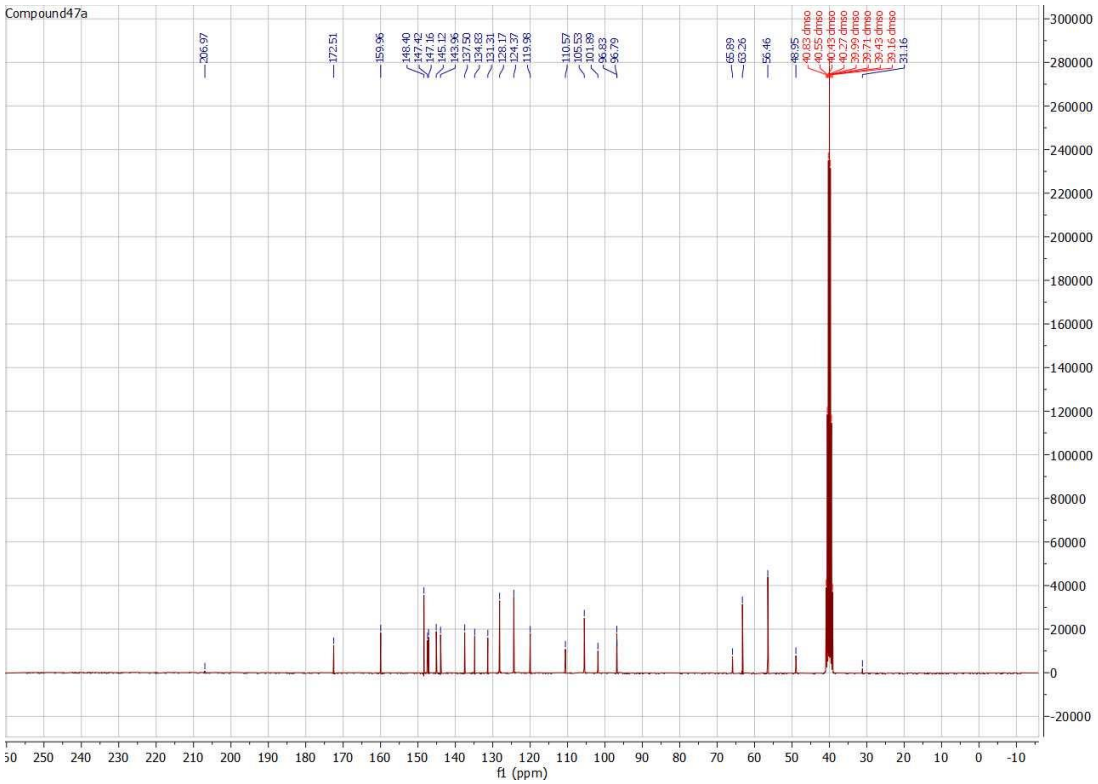


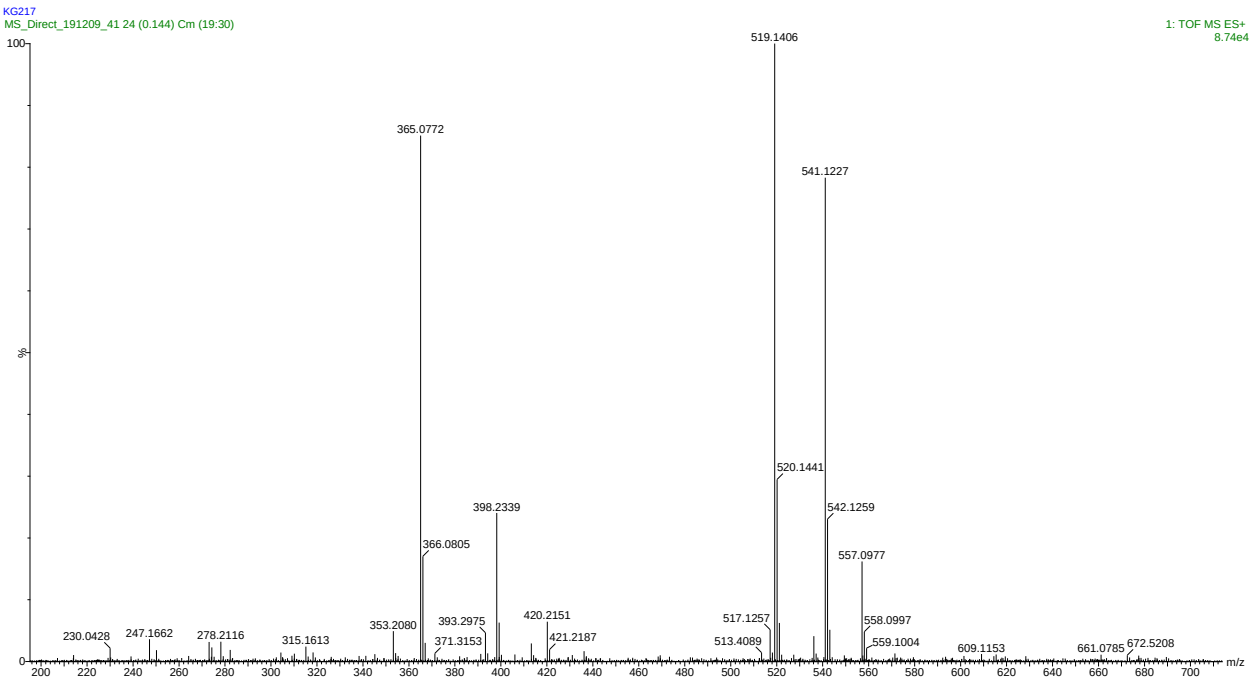


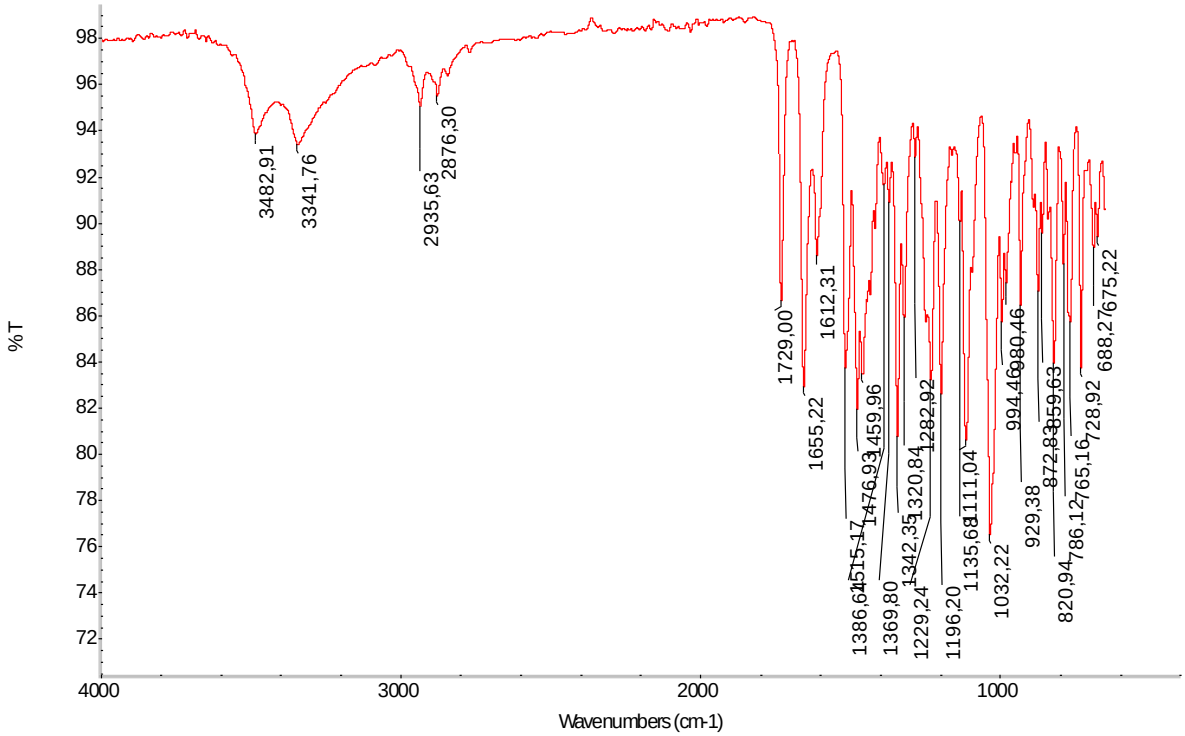


7.1.2.5 *Analysis of 9-(4-hydroxy-3,5-dimethoxyphenyl)-5-(4-nitrobenzyl)-6,9-dihydro [1,3]dioxolo [4,5-g]furo[3,4-b]quinolin-8(5H)-one (37a)*

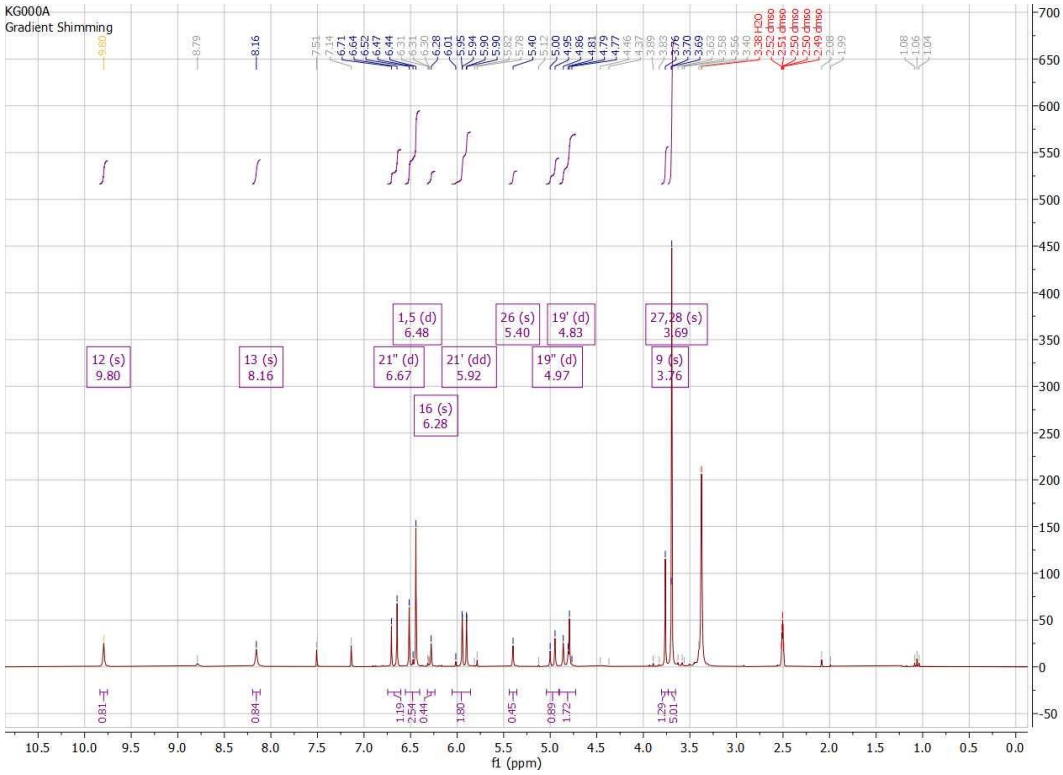


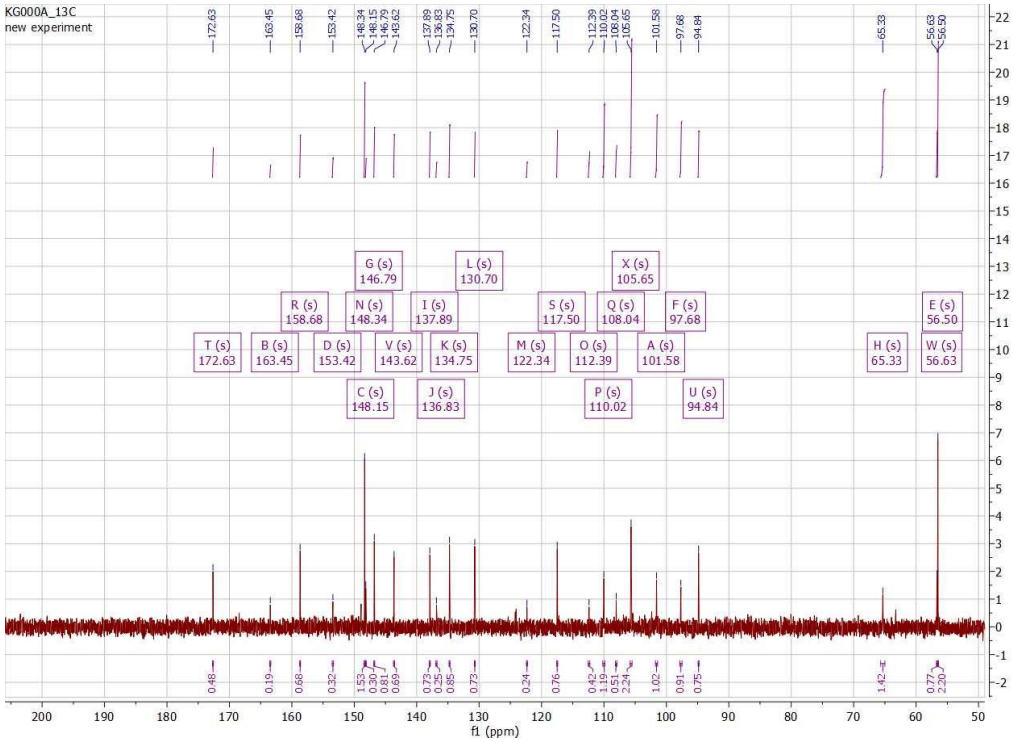


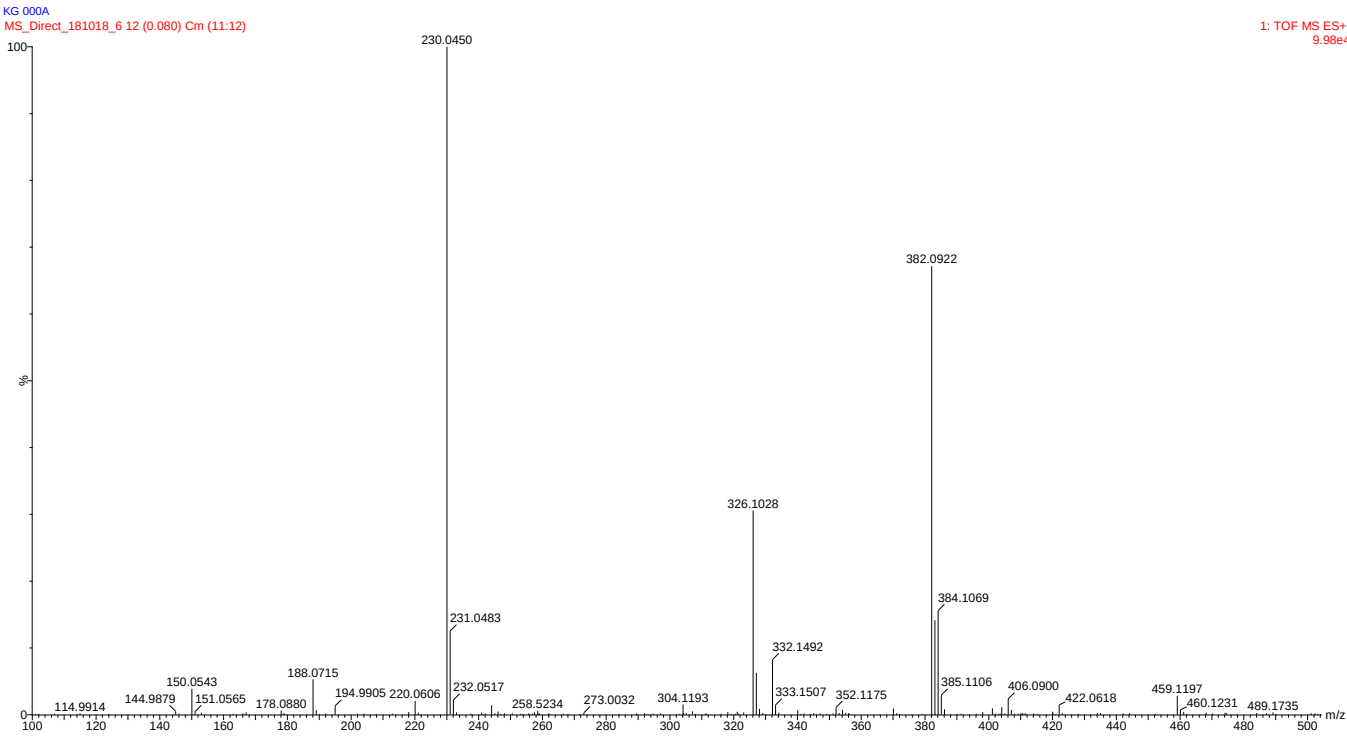


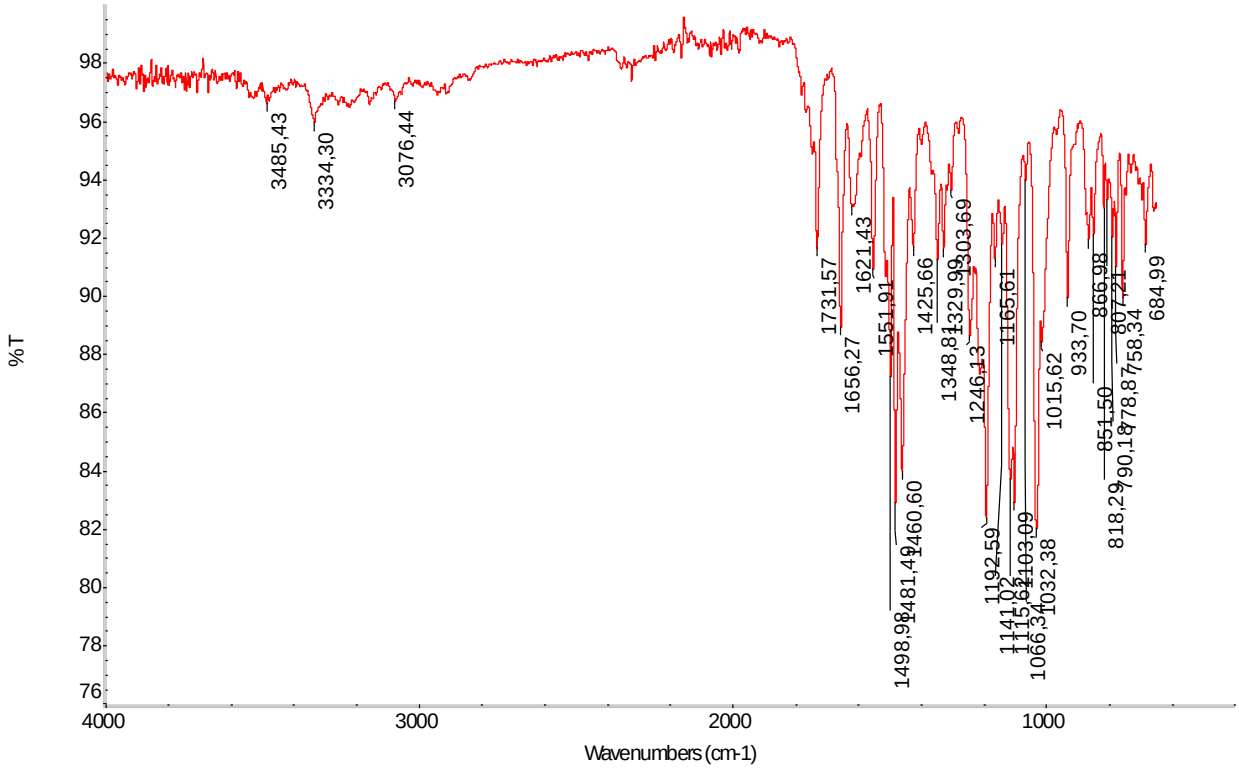


7.1.2.6 *Analysis of 9-(4-hydroxy-3,5-dimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one (38a)*

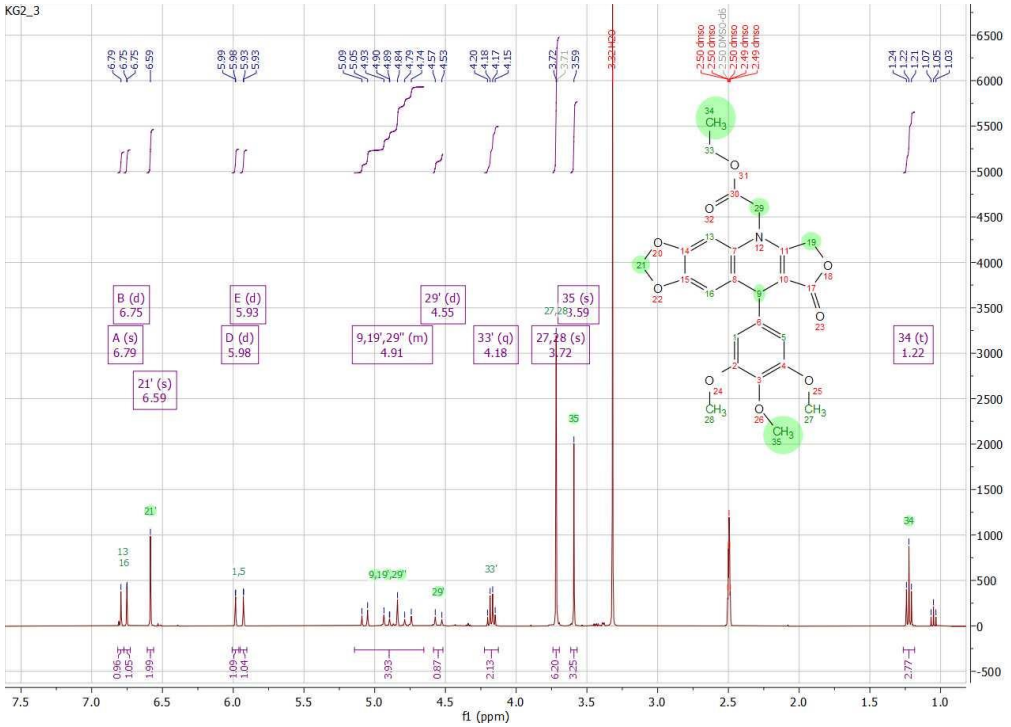


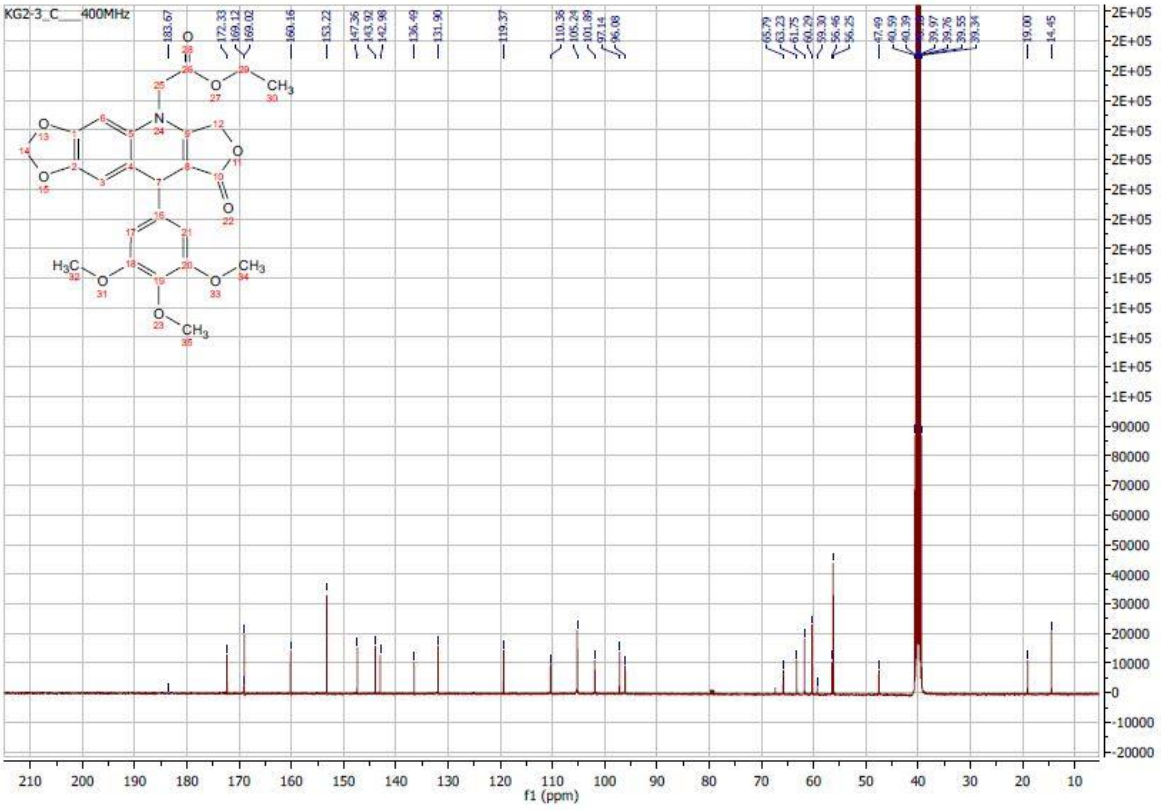


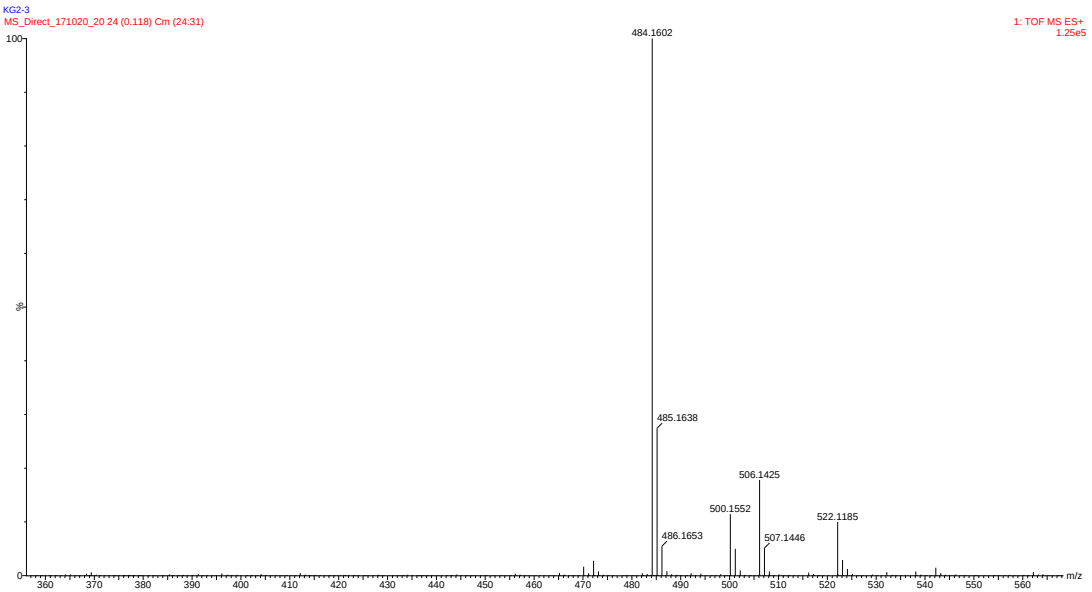


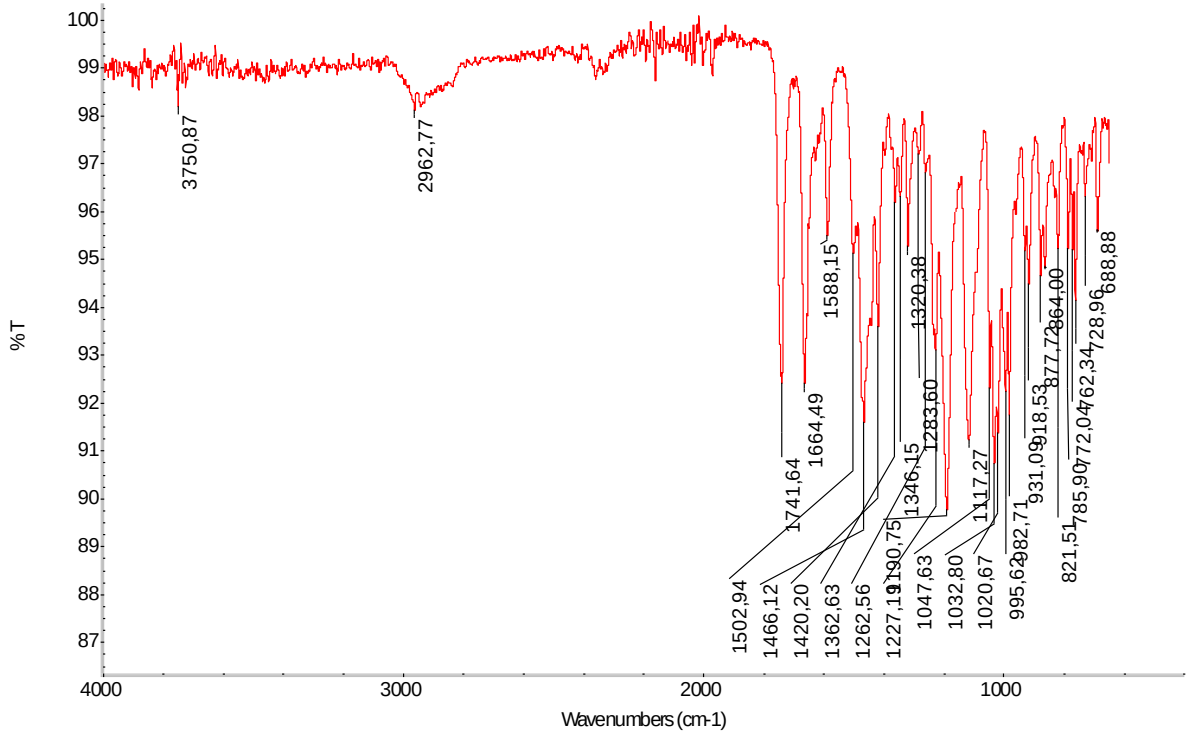


7.1.2.7 *Analysis of Ethyl 2-[8-oxo-9-(3,4,5-trimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5g]furo[3,4-b]quinolin-5(8H)-yl]acetate (33b)*

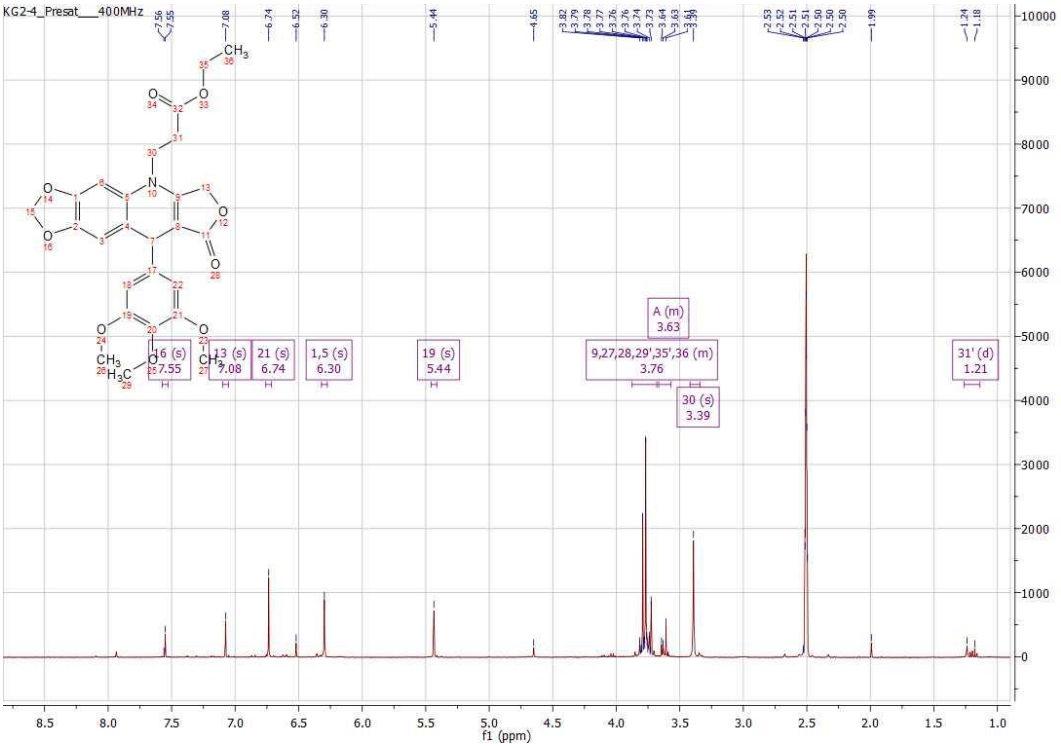


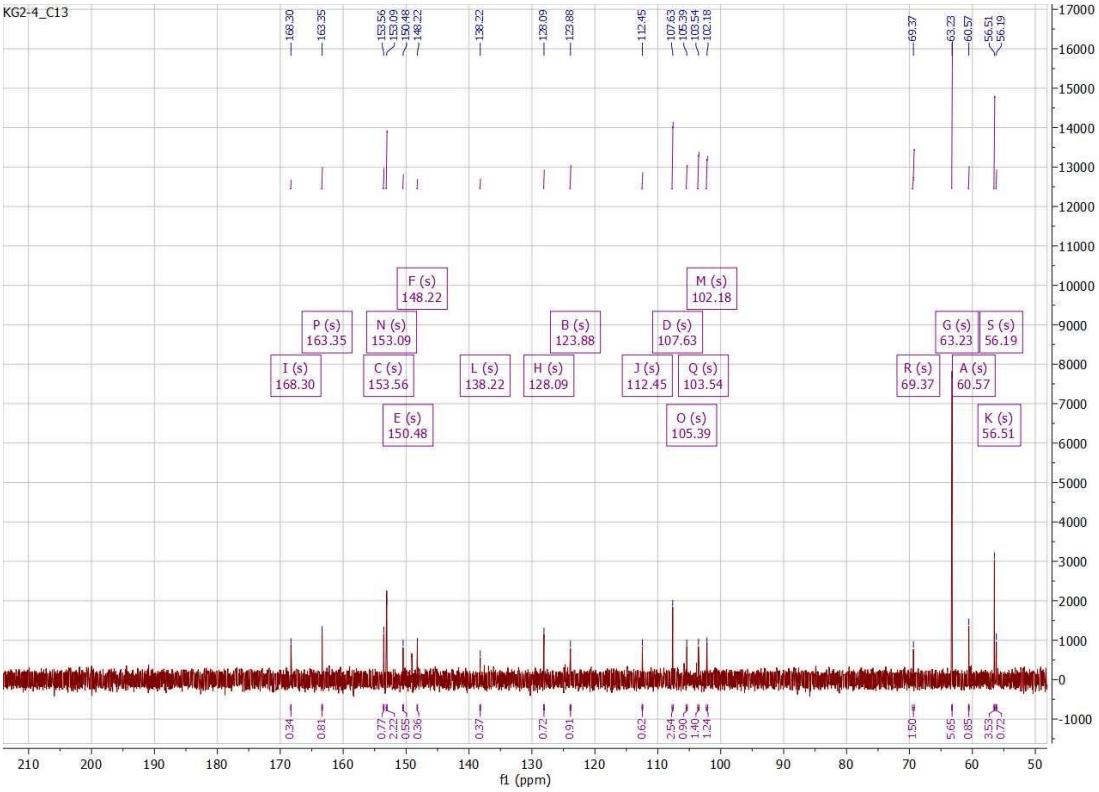


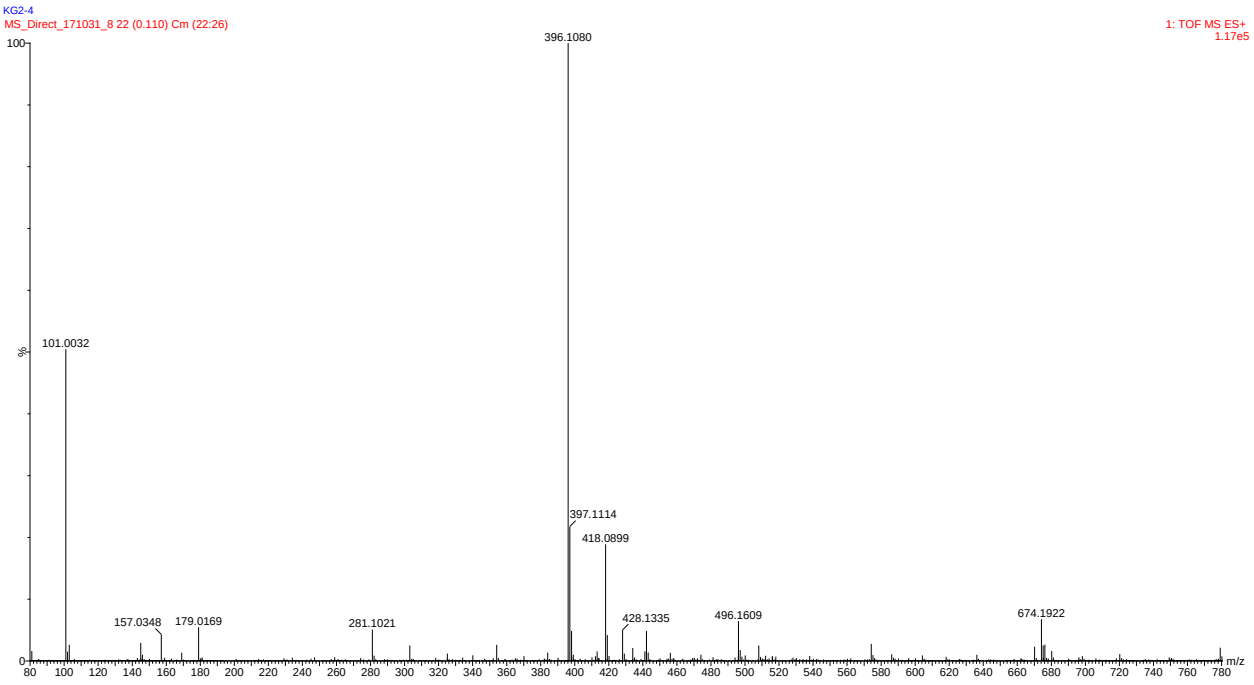


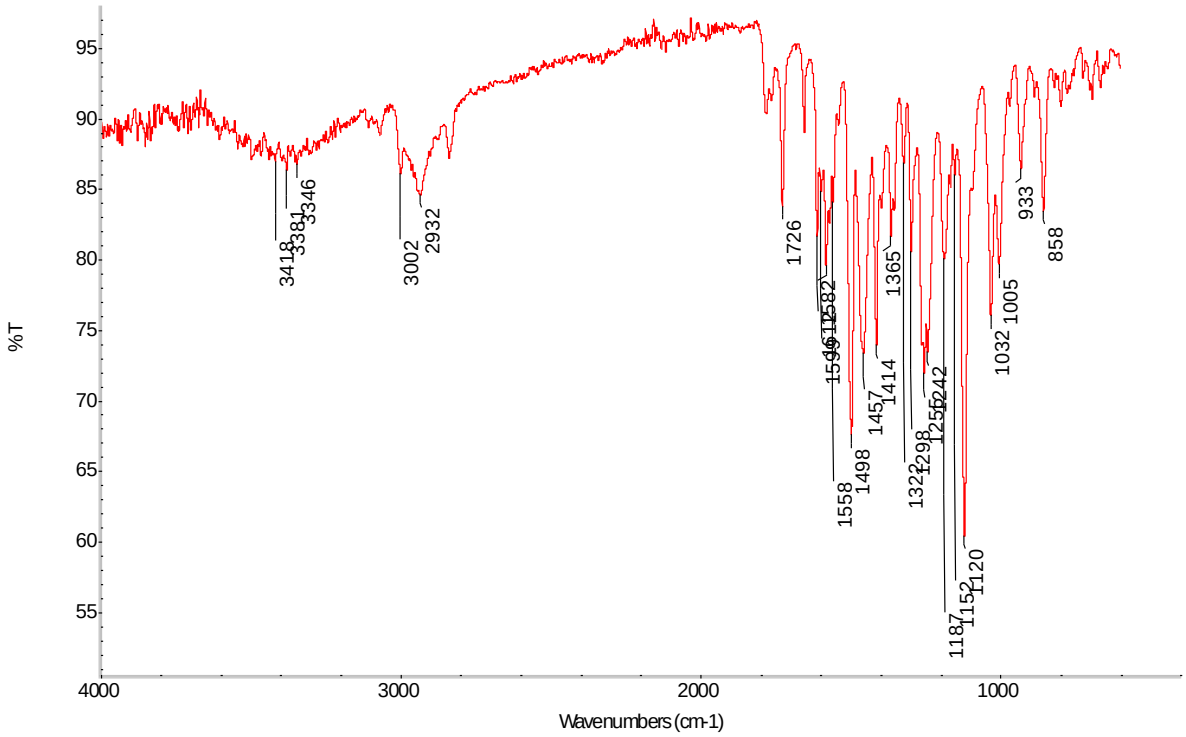


7.1.2.8 *Analysis of ethyl 3-(8-oxo-9-(3,4,5-trimethoxyphenyl)-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-5(6H,8H,9H)-yl)propanoate (34b)*

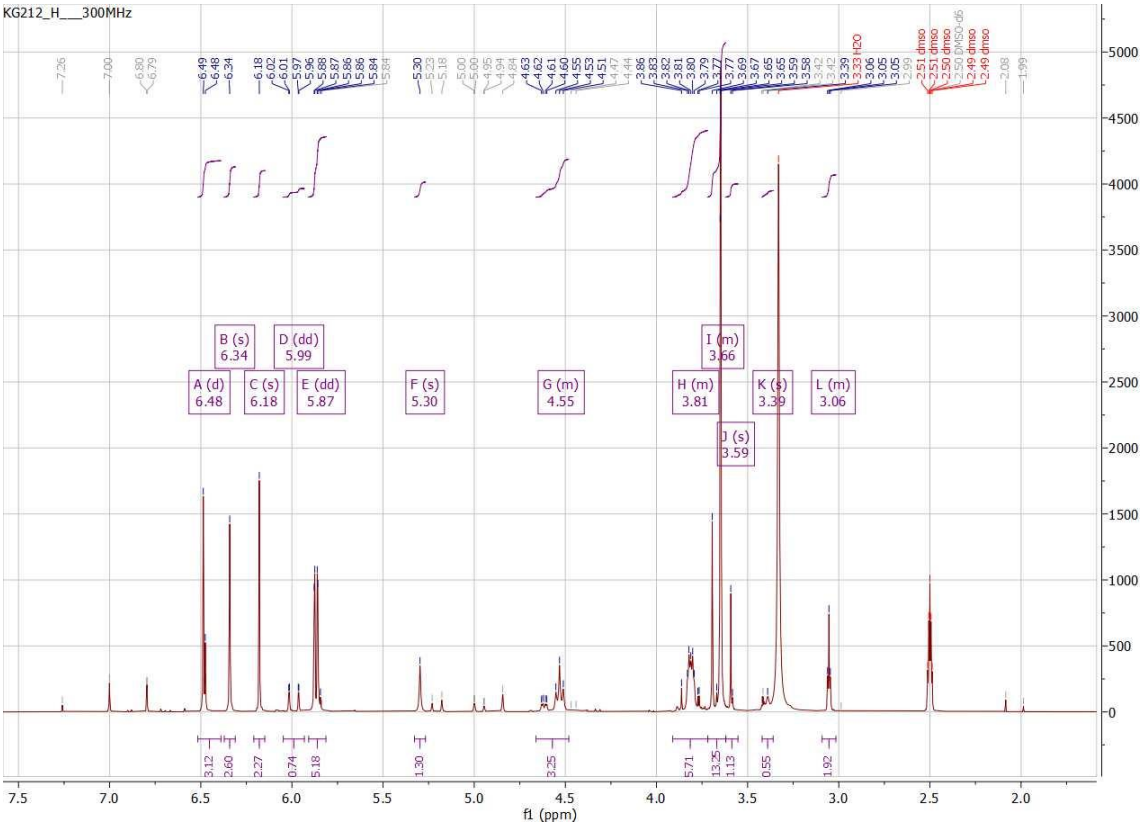


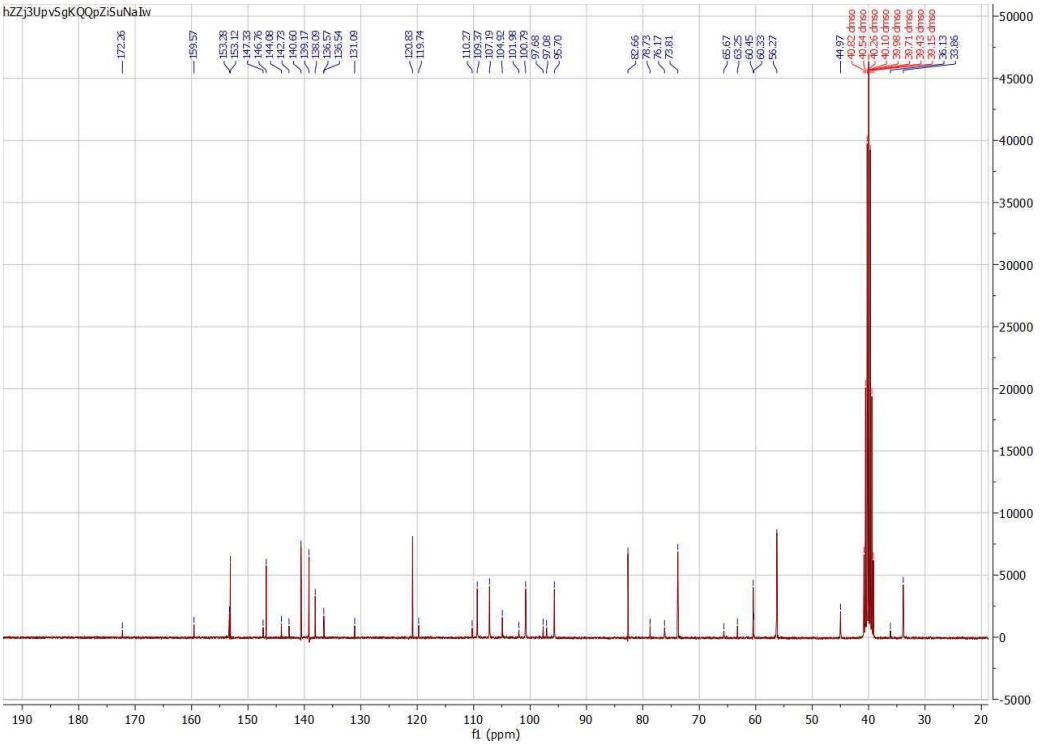


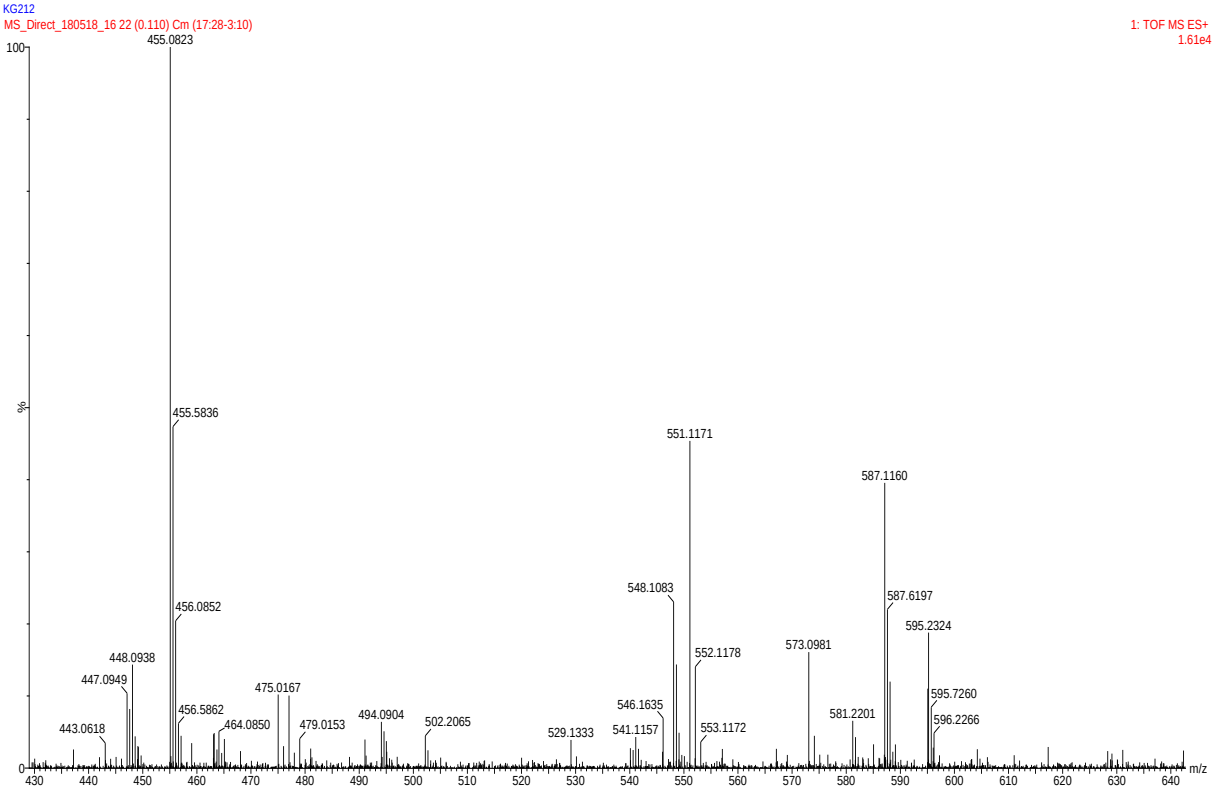


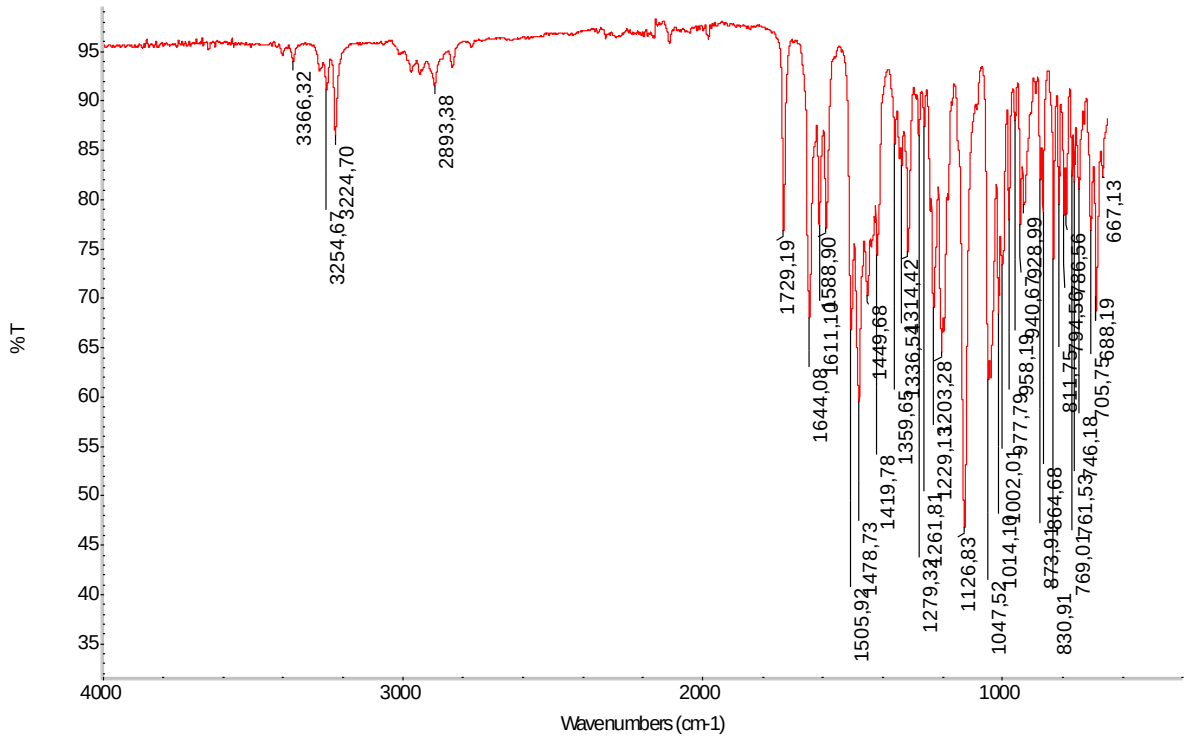


7.1.2.9 *Analysis of unknown compound (35b)*

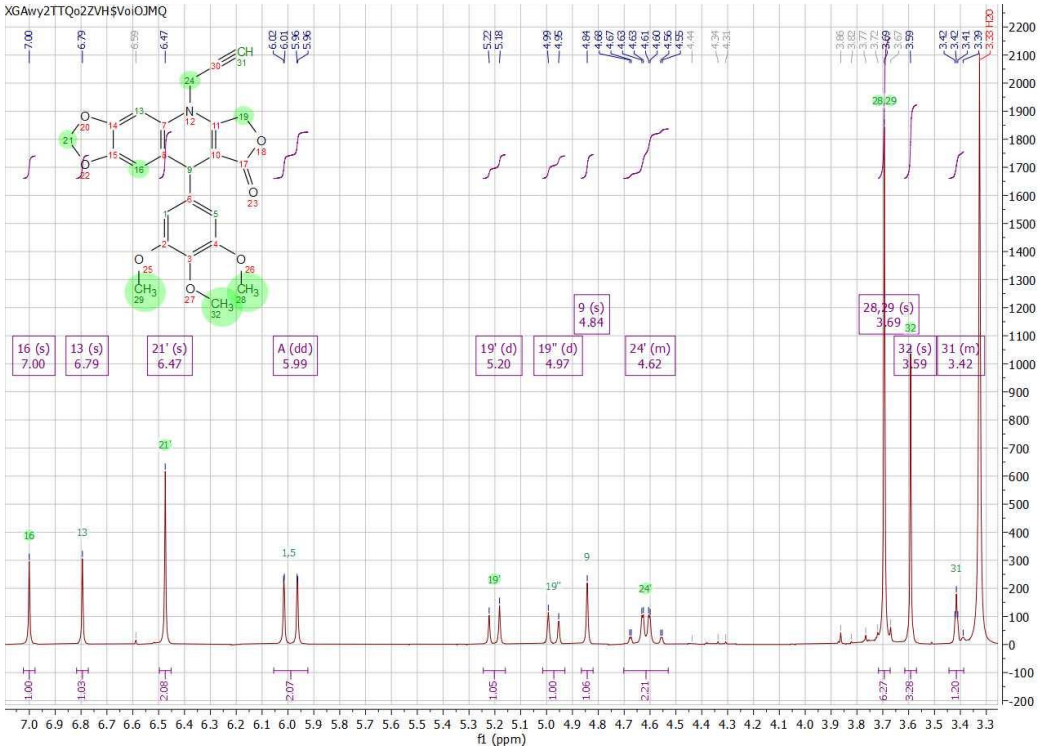


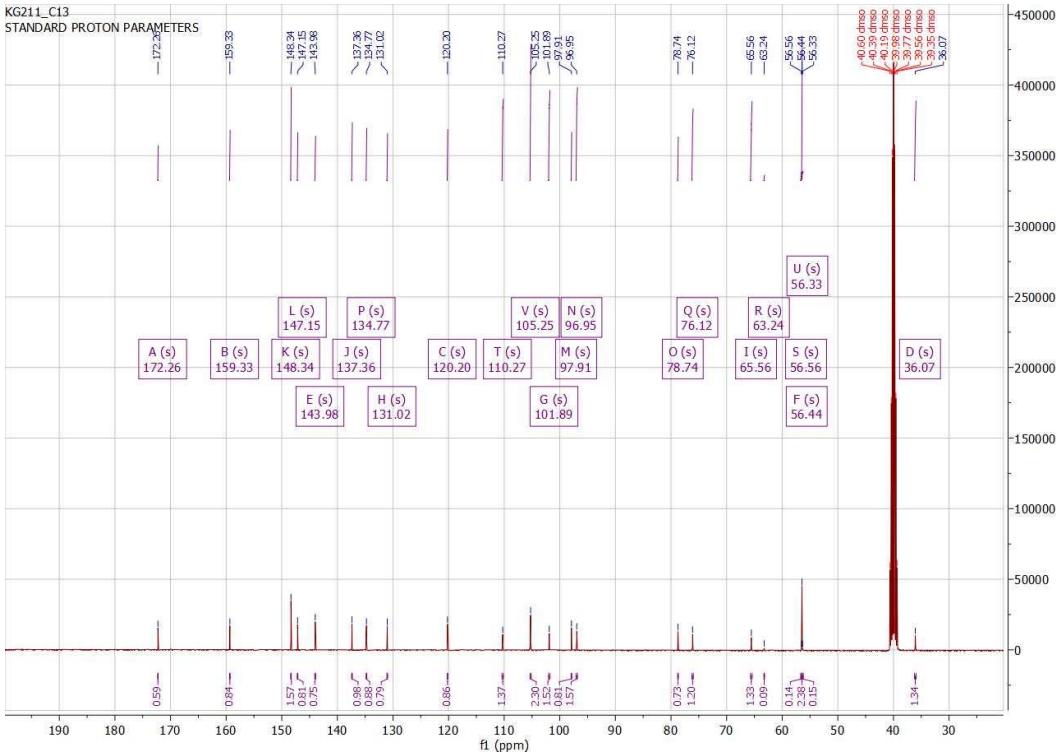


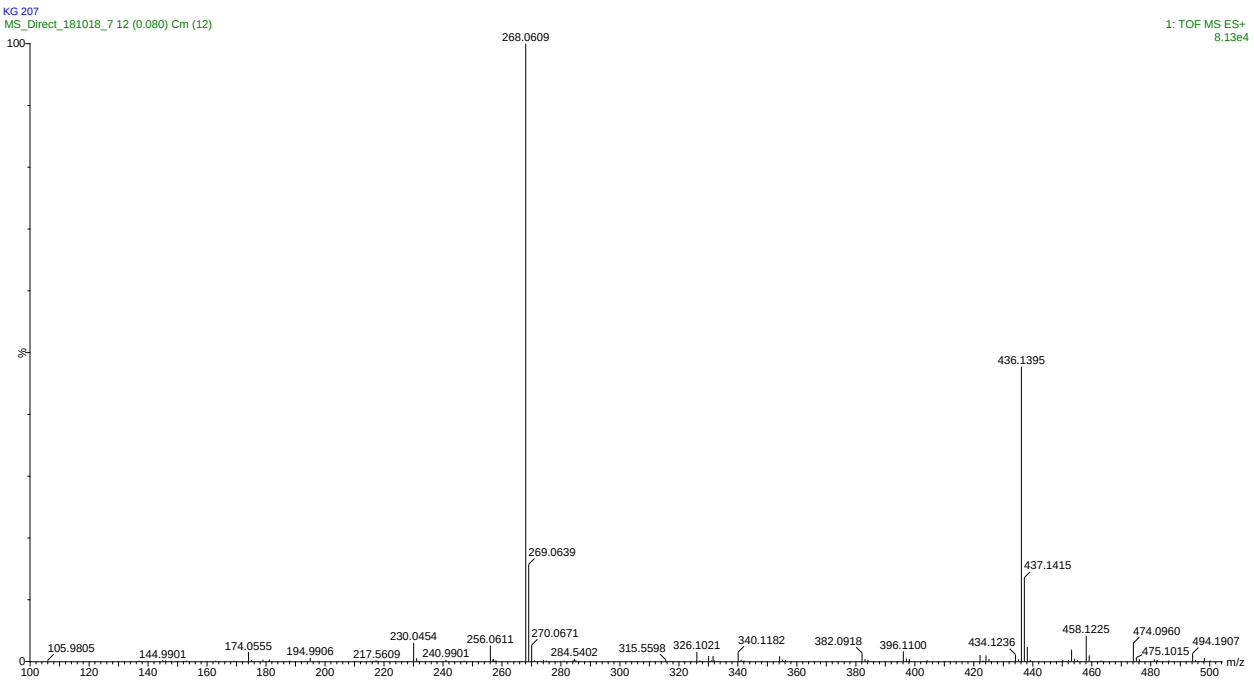


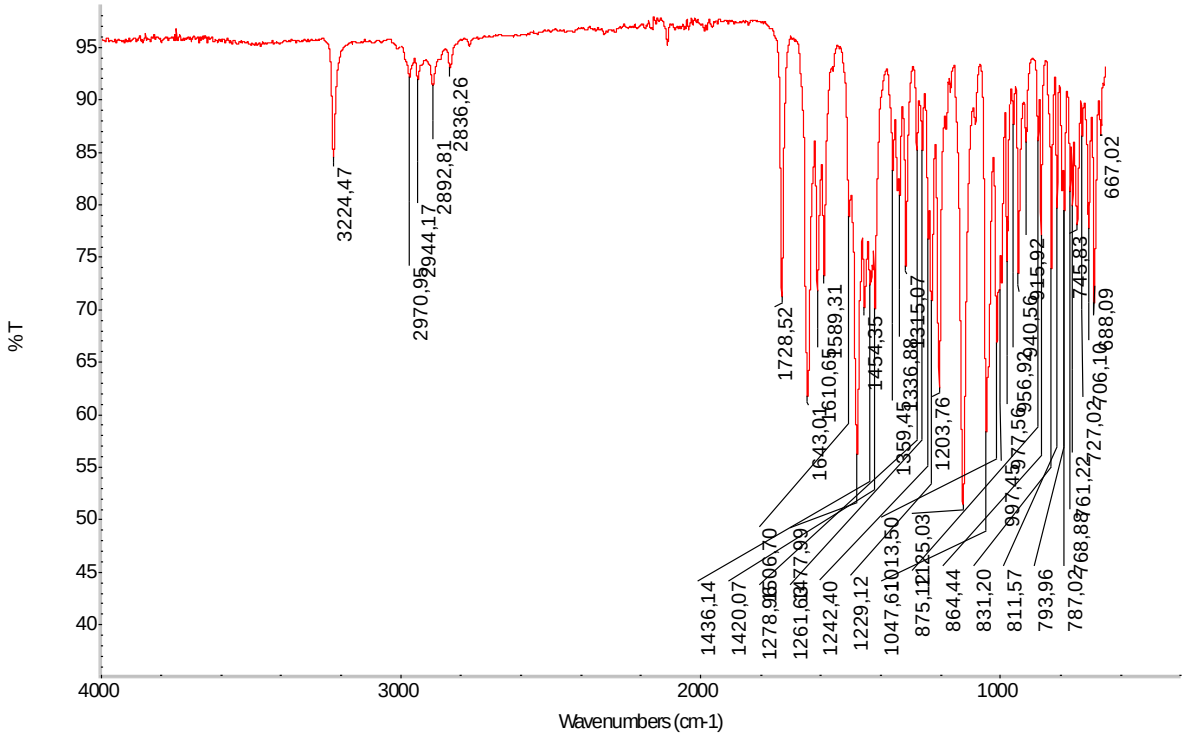


7.1.2.10 *Analysis of 5-(prop-2-yn-1-yl)-9-(3,4,5-trimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one (36b)*

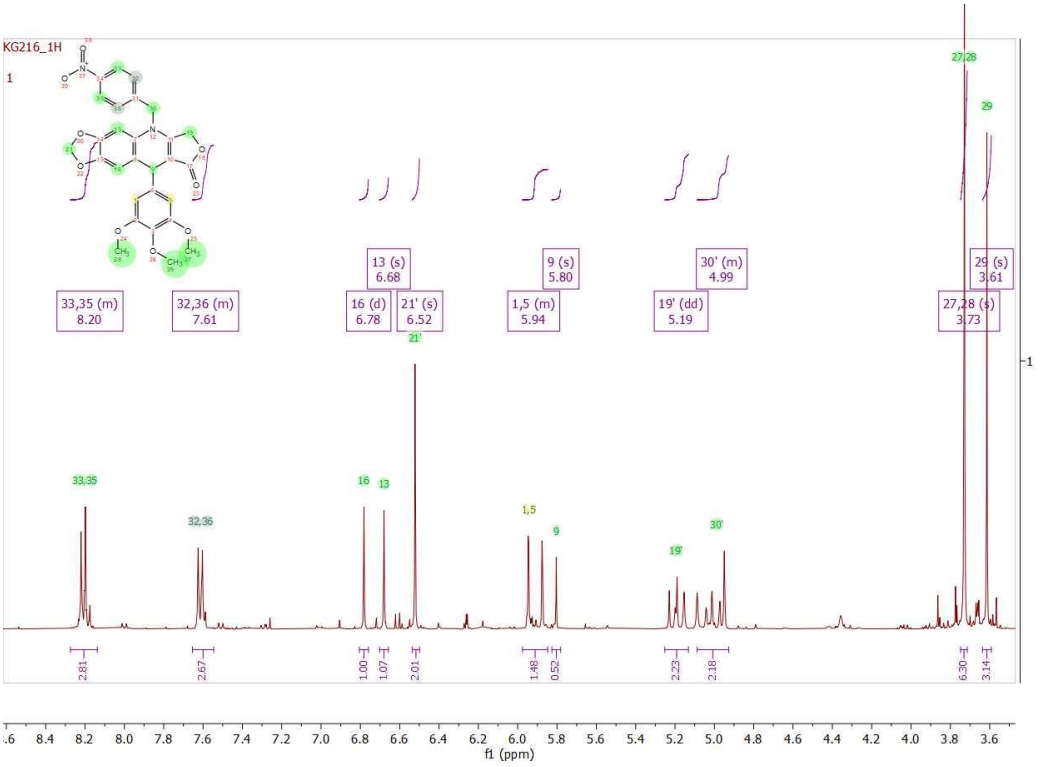


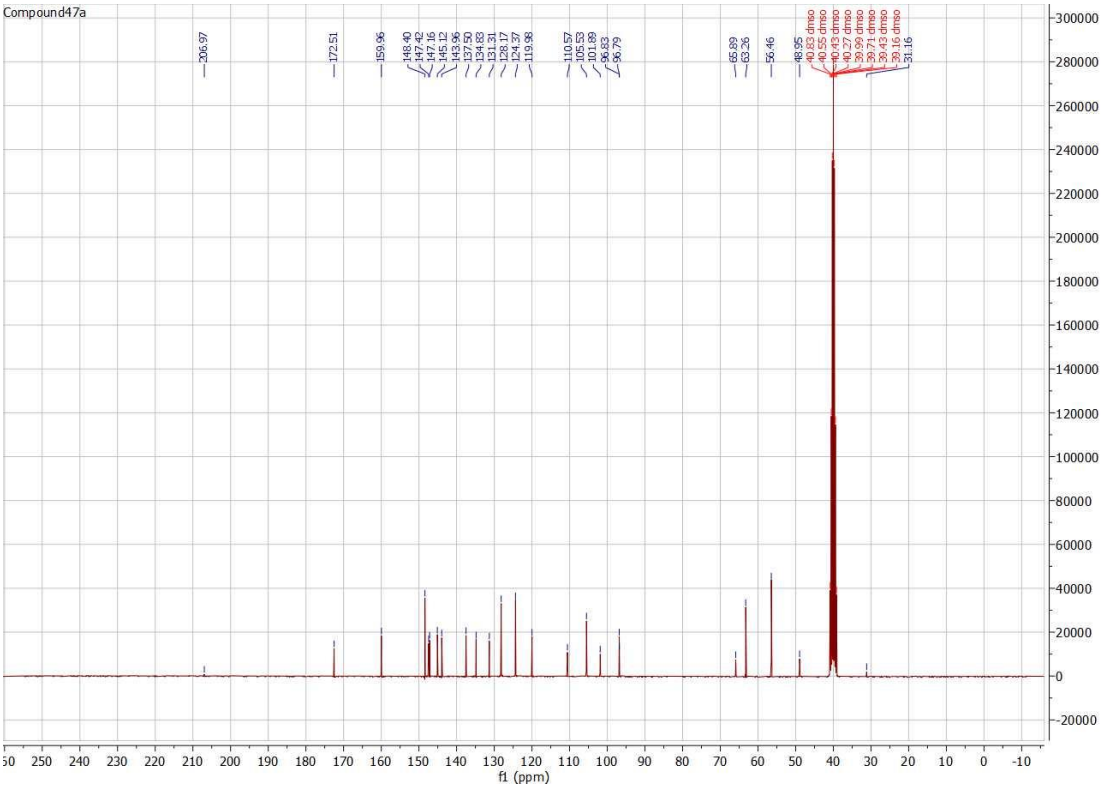


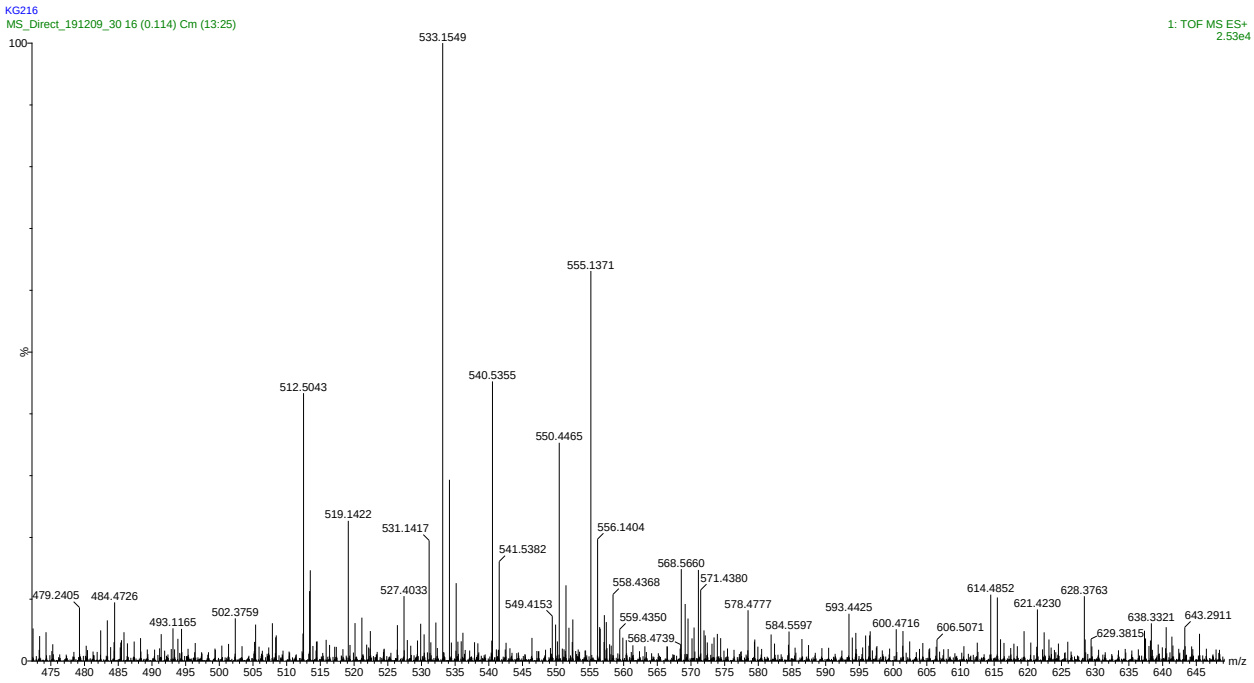




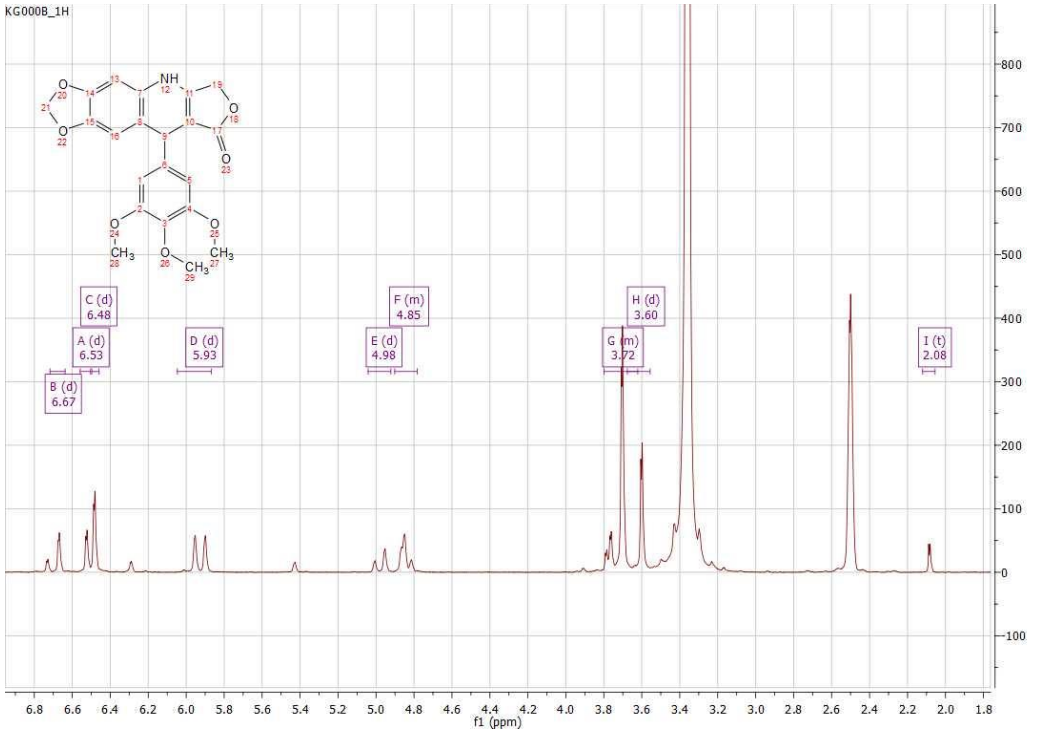
7.1.2.11 *5-(4-nitrobenzyl)-9-(3,4,5-trimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one(37b)*

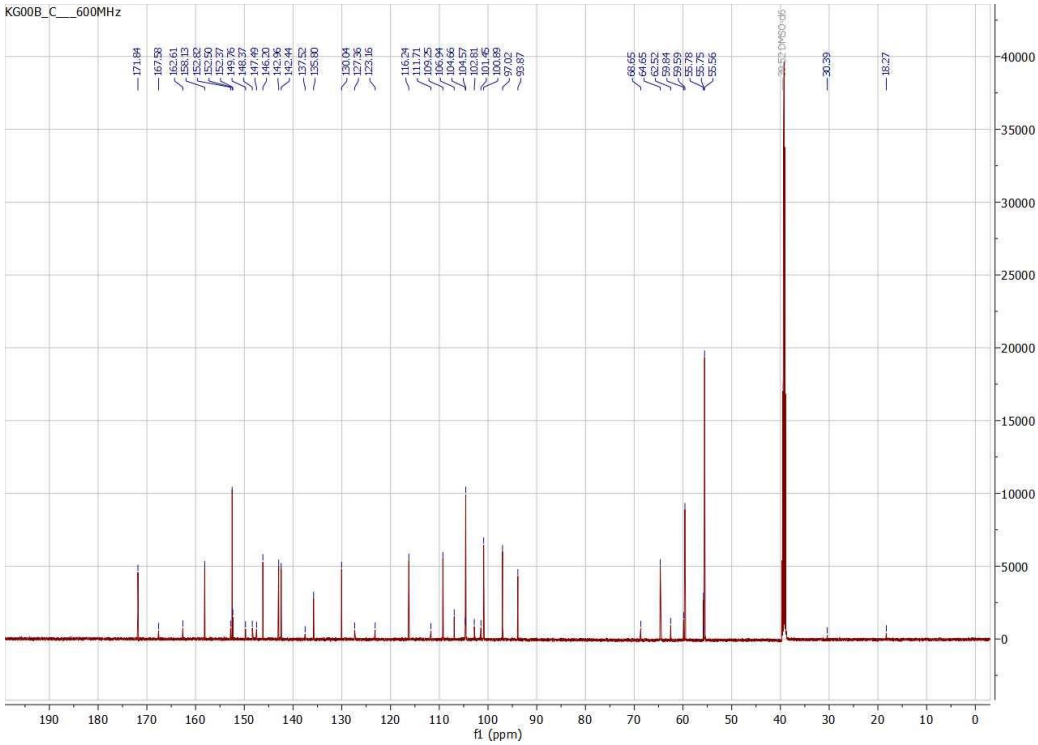


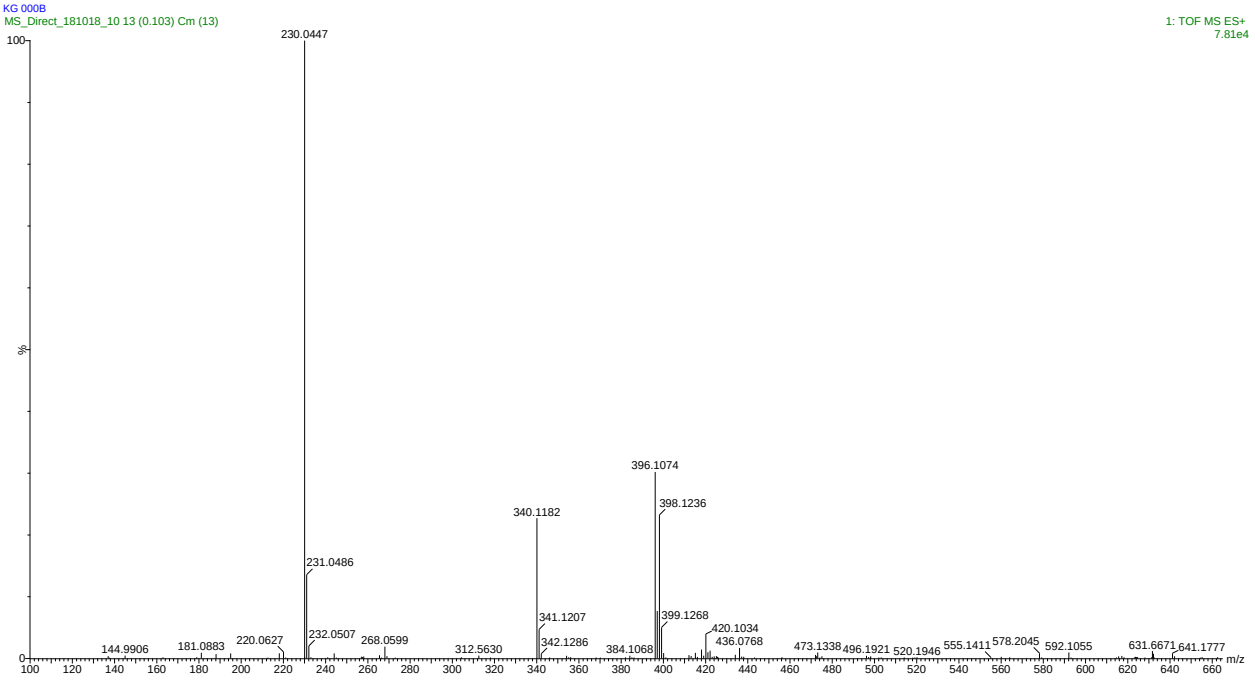


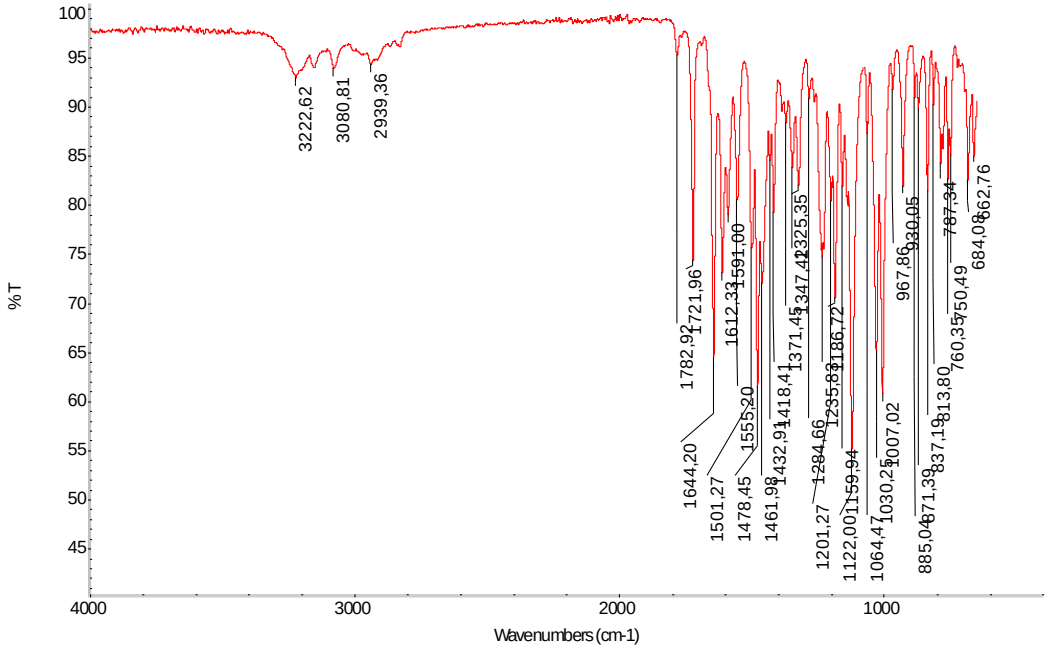


7.1.2.12 *Analysis of 9-(3,4,5-trimethoxyphenyl)-6,9-dihydro-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-8(5H)-one (38b)*



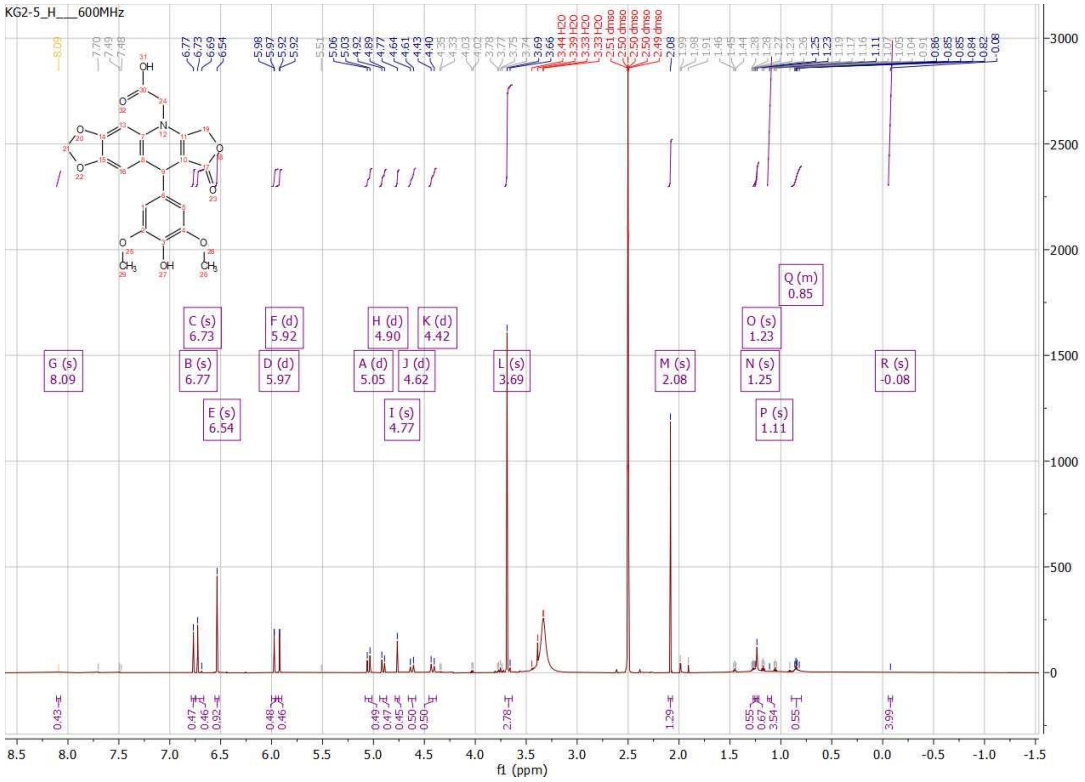


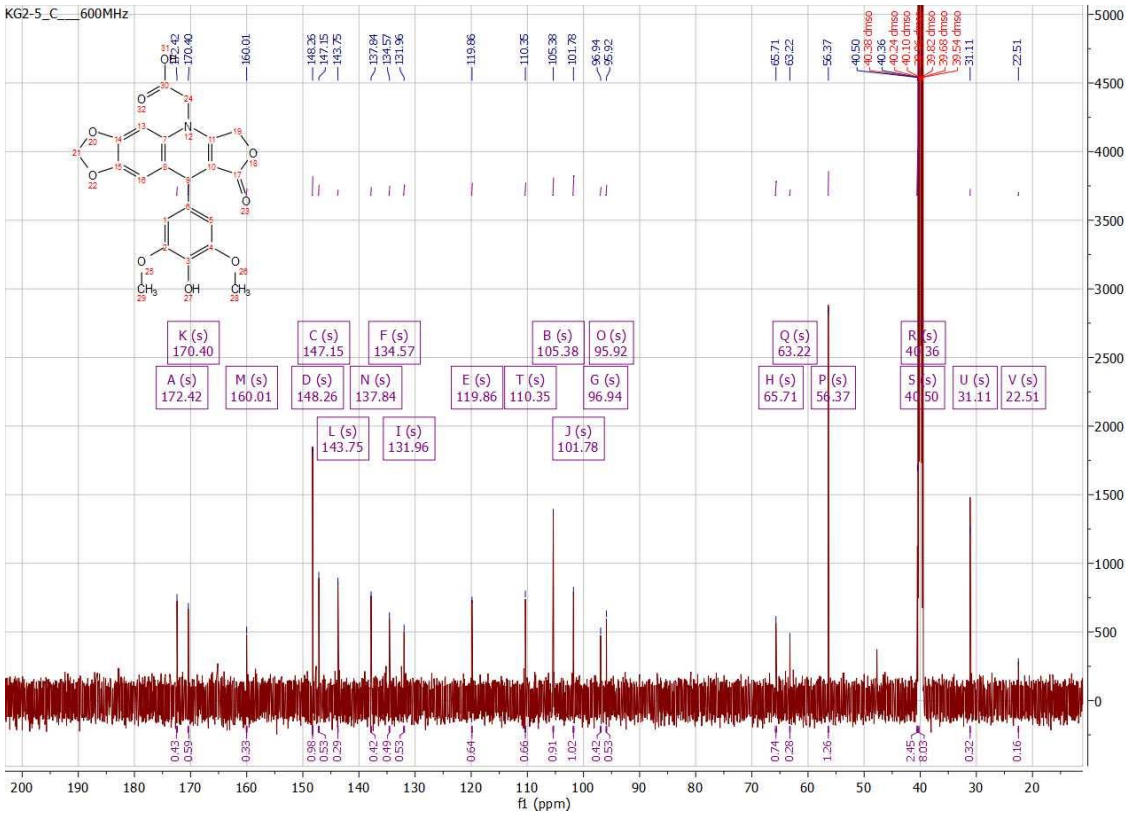


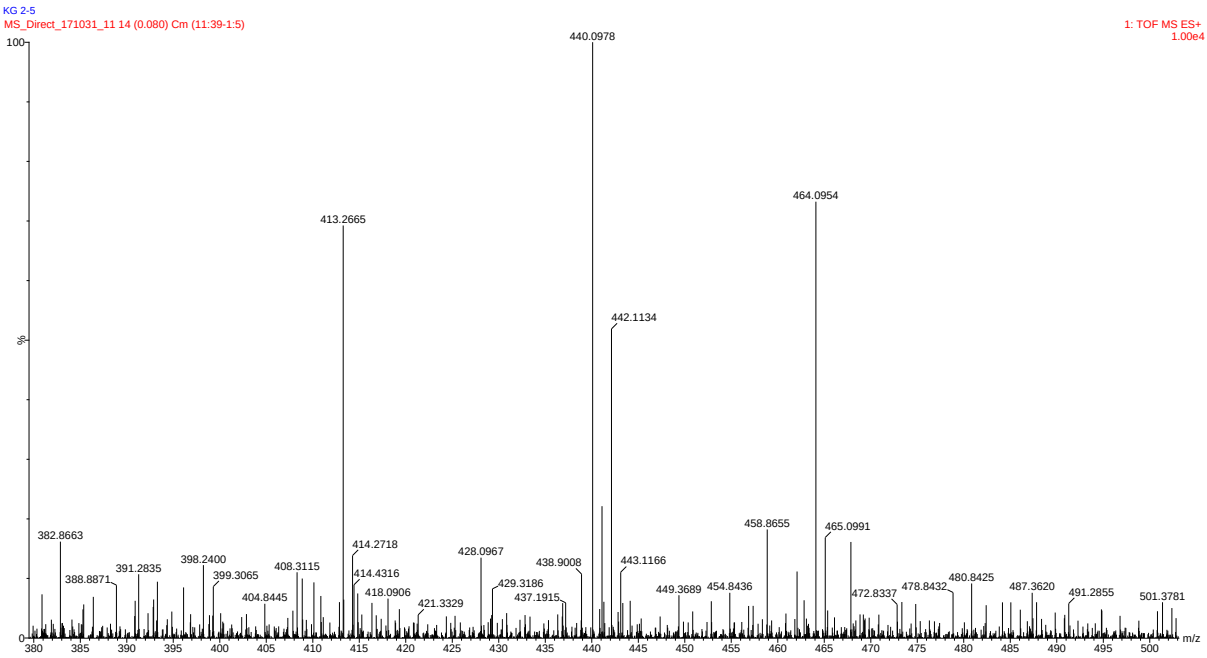


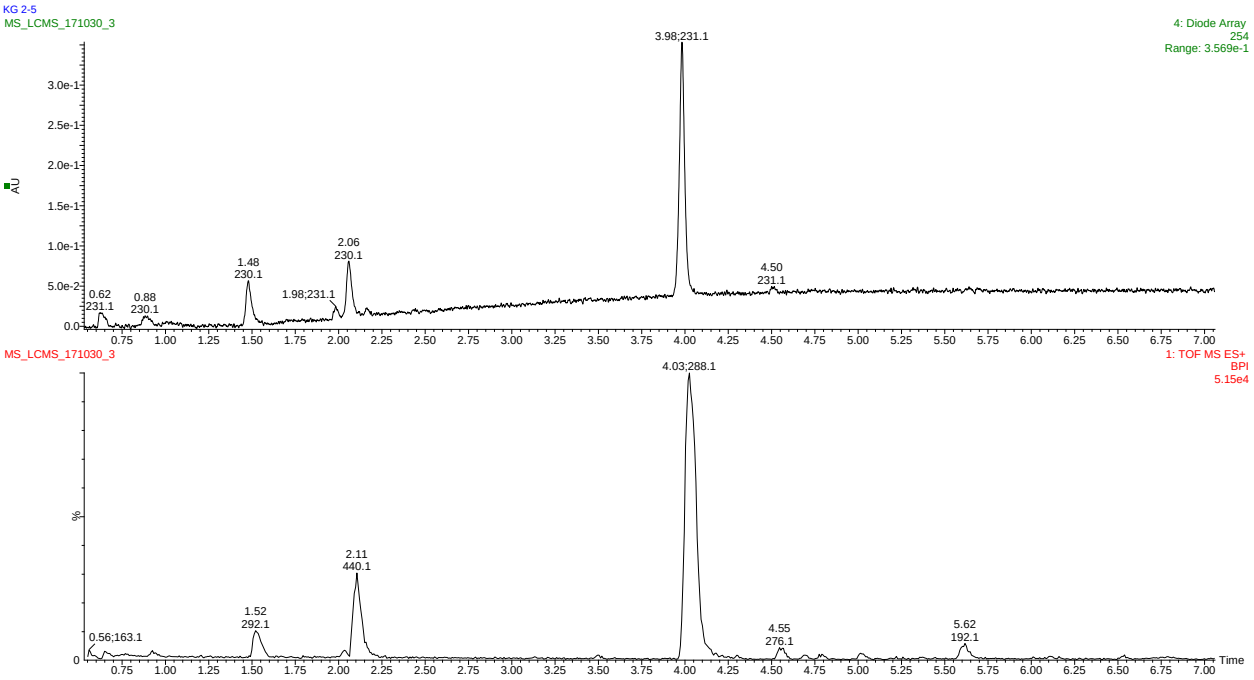
7.1.3 Hydrolysis of 33a and 33b

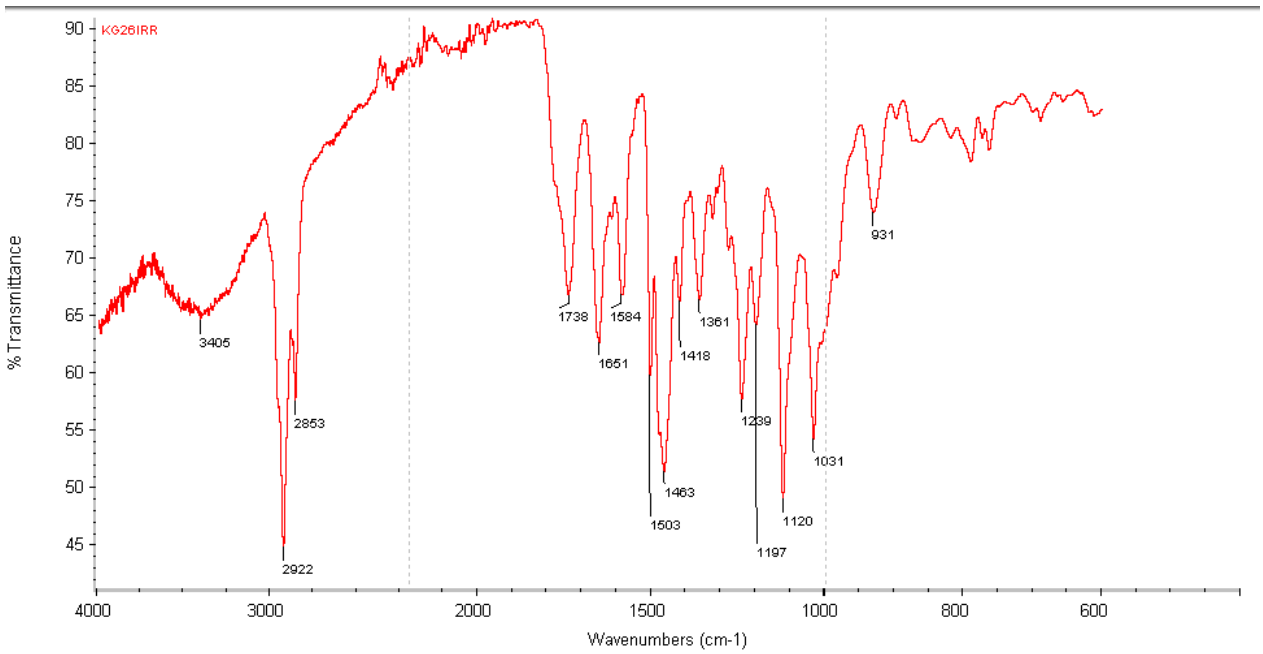
7.1.3.1 *Compound 2-(9-(4-hydroxy-3,5-dimethoxyphenyl)-8-oxo-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-5(6H,8H,9H)-yl)acetic acid (39a)*



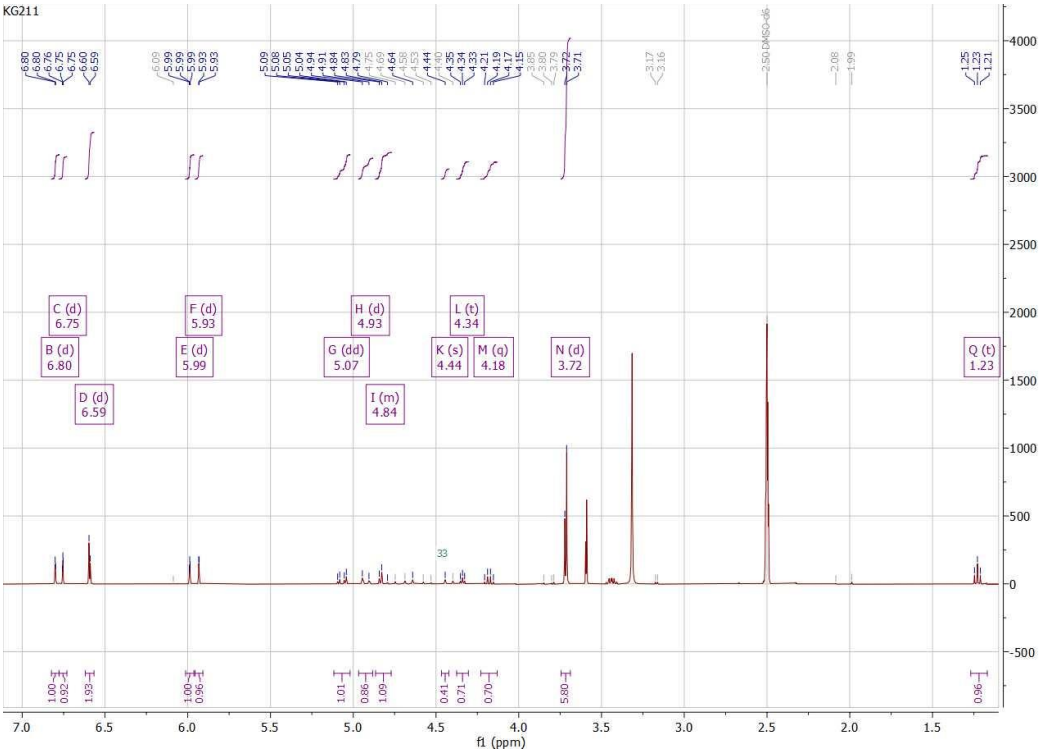


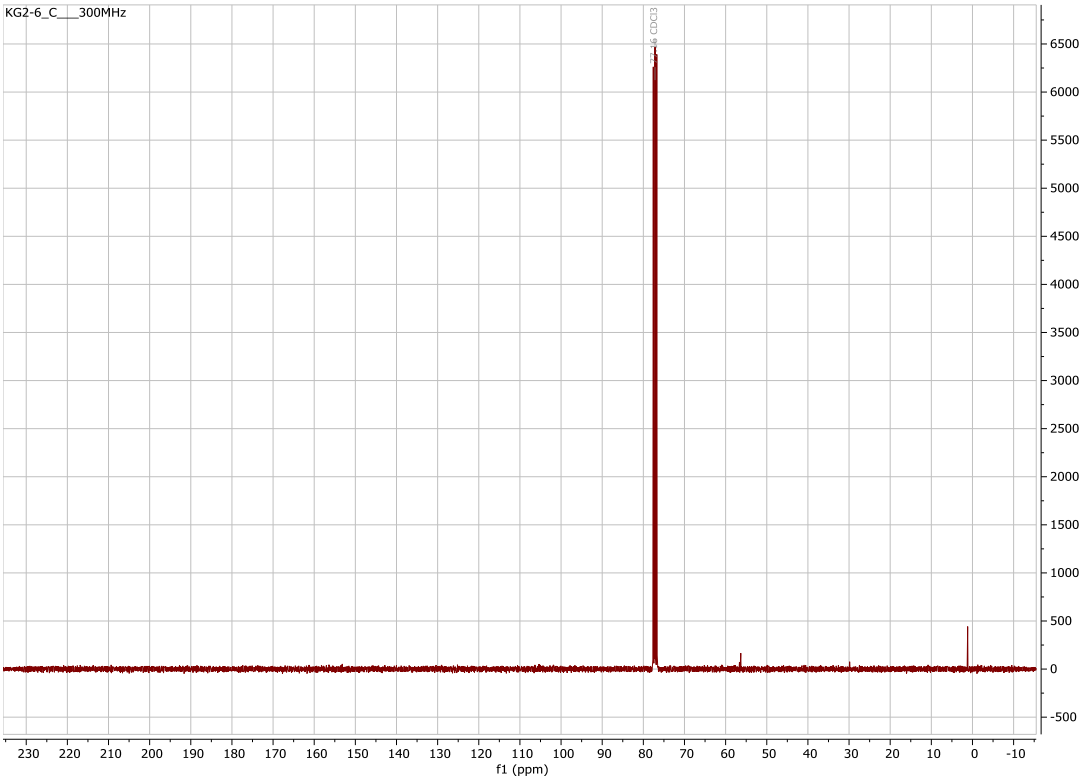


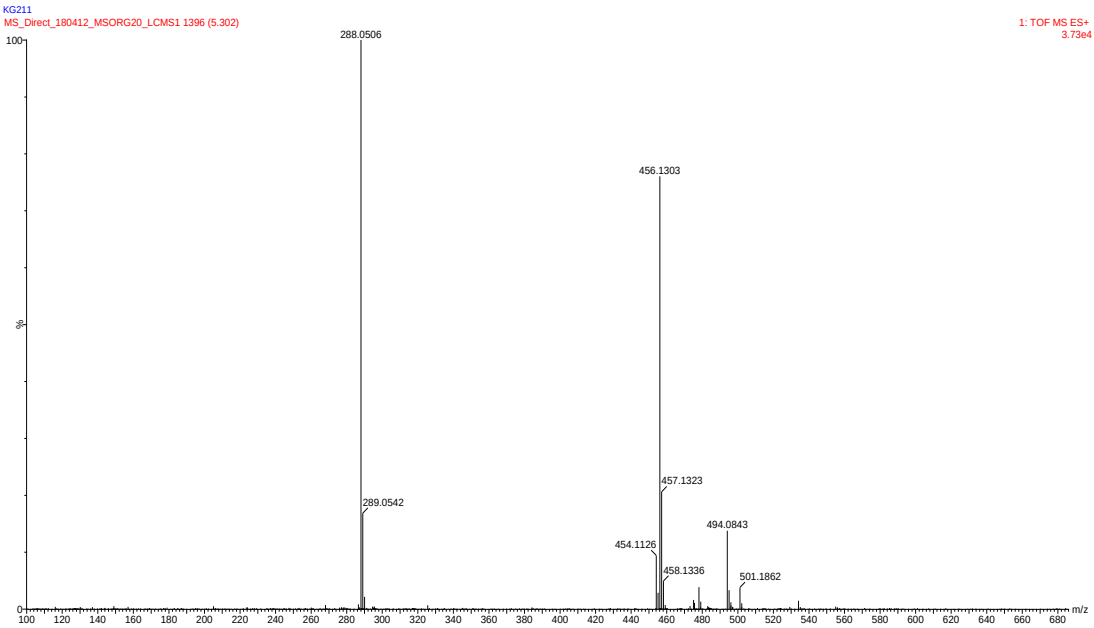


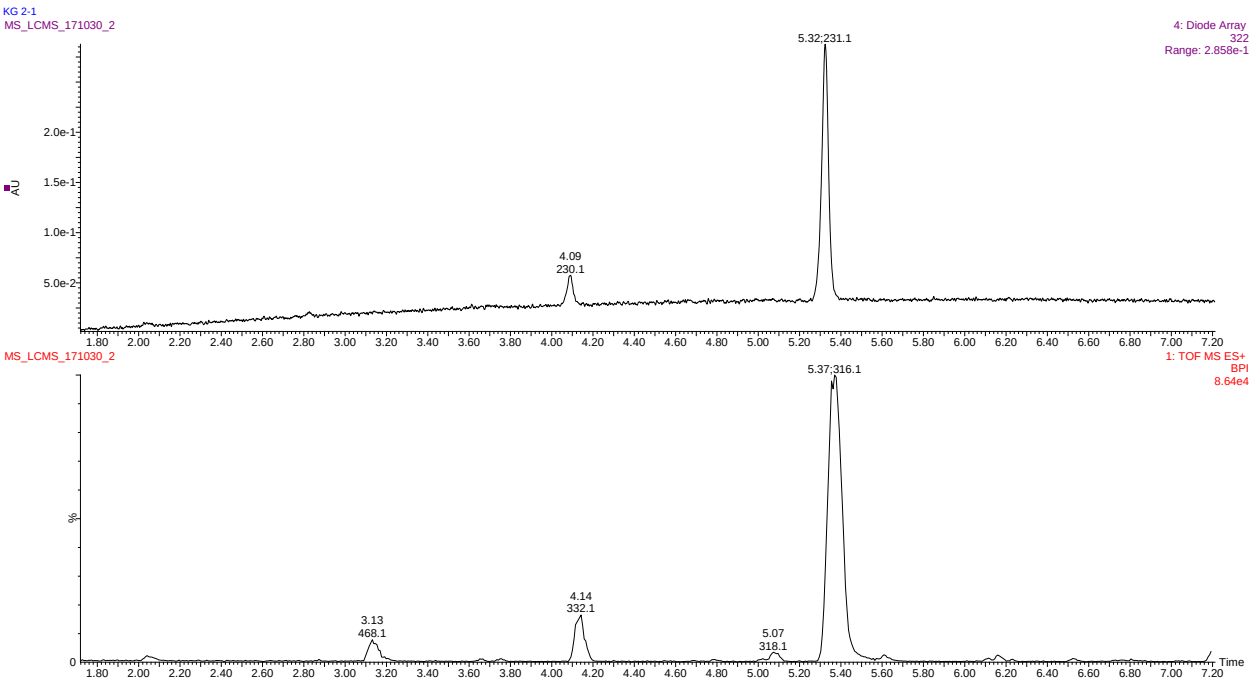


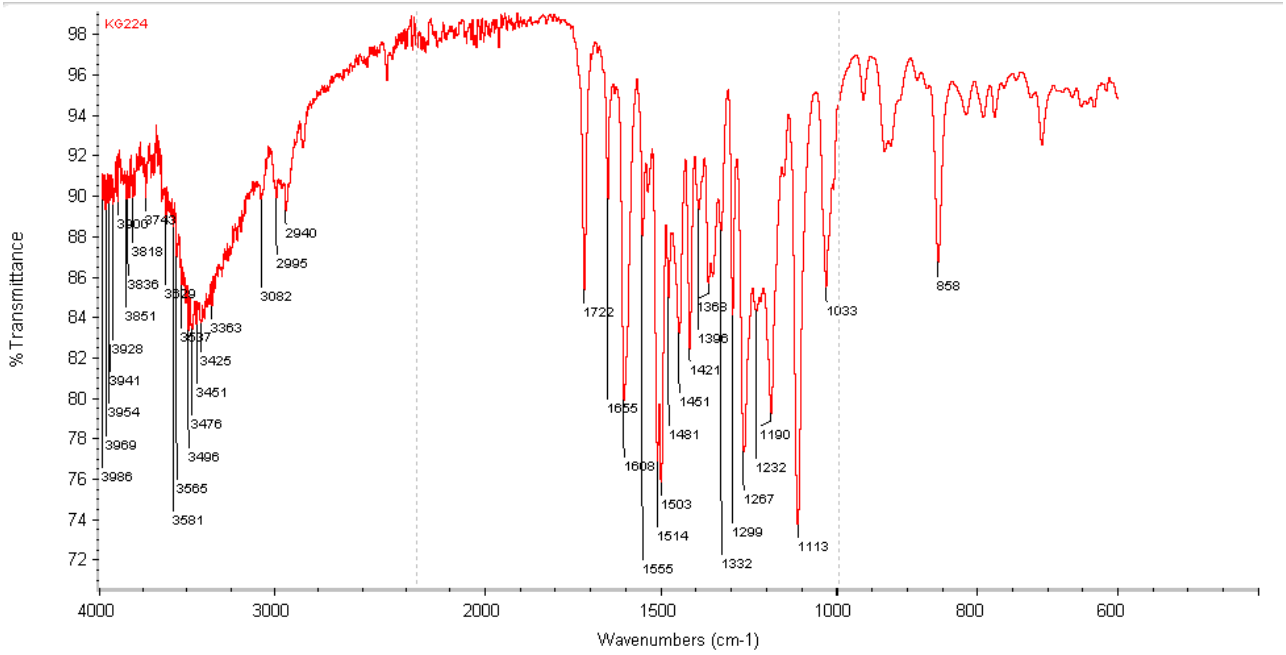
7.1.3.2 *Compound 2-(8-oxo-9-(3,4,5-trimethoxyphenyl)-[1,3]dioxolo[4,5-g]furo[3,4-b]quinolin-5(6H,8H,9H)-yl)acetic acid (39b)*











Supplementary information-Part II

Table of Contents

8.1 Click reactions used in the synthesis of Library 2

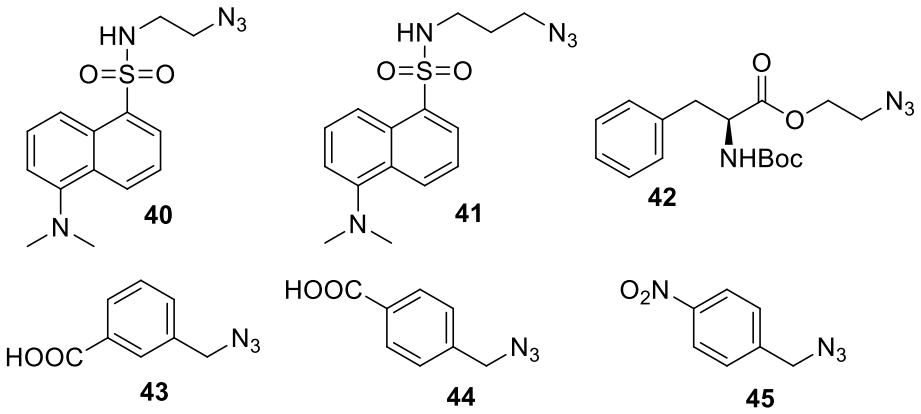
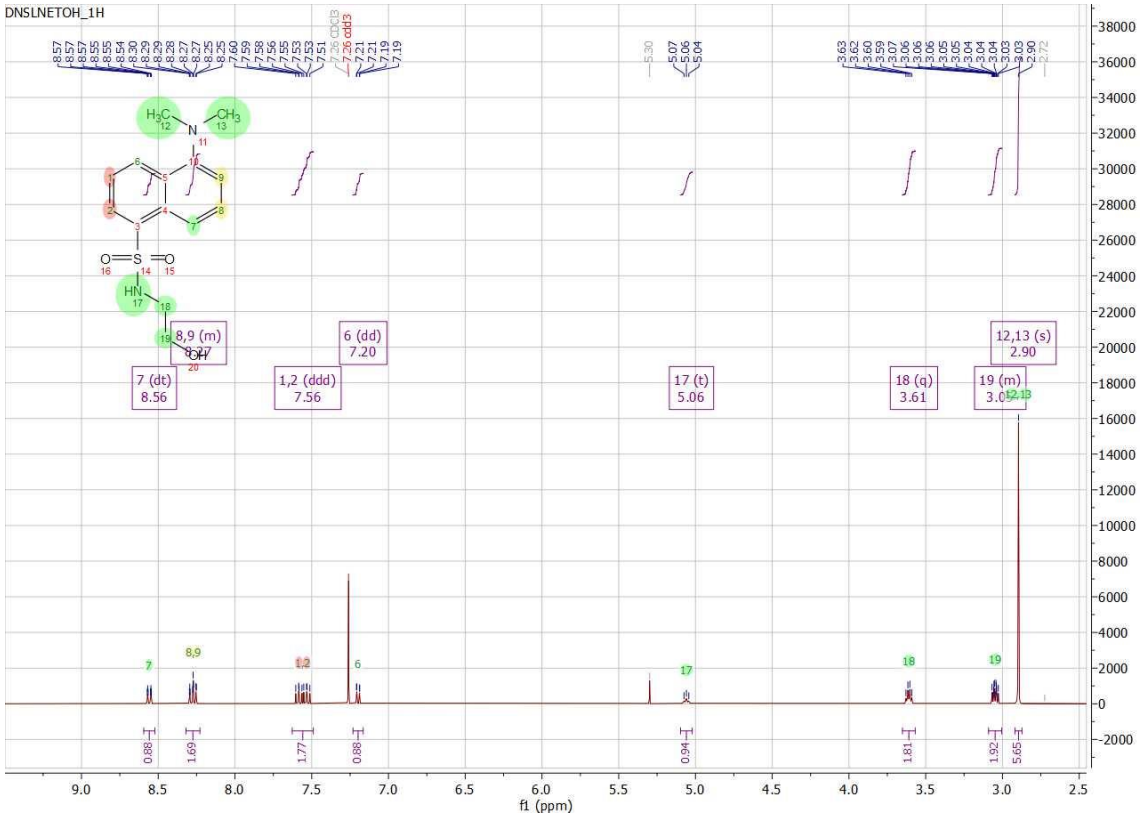


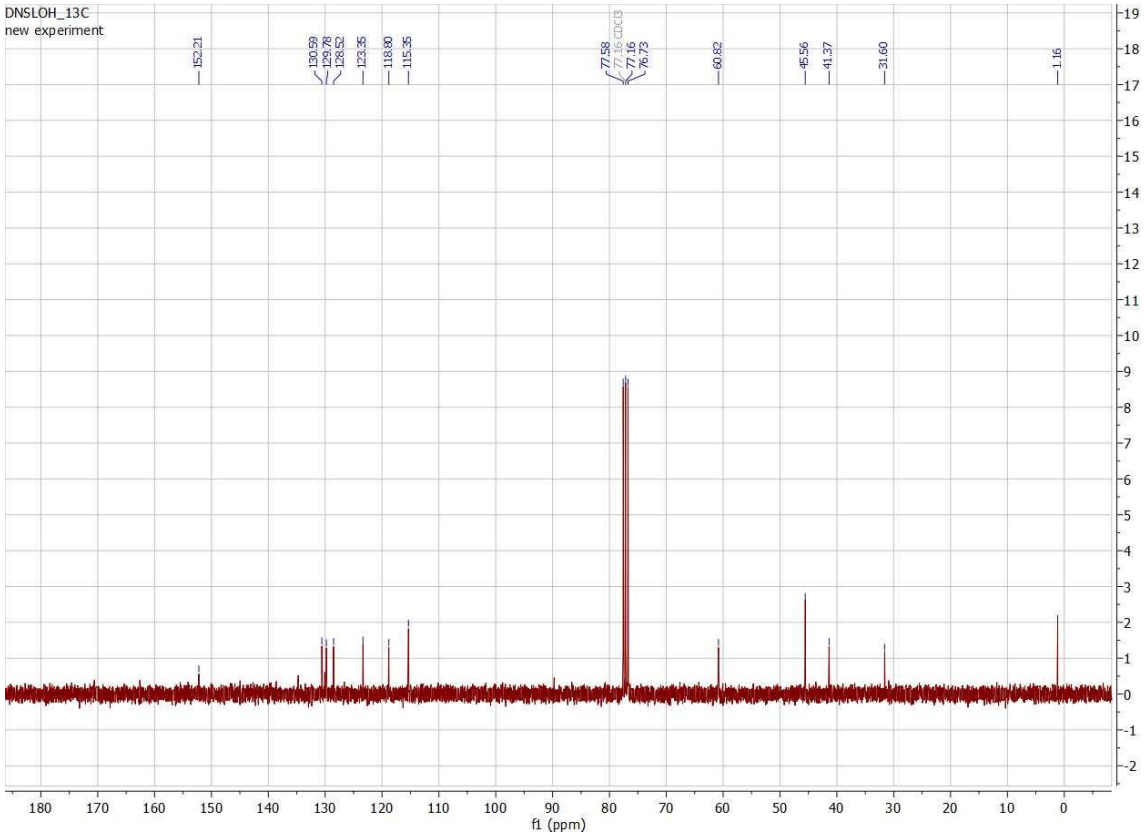
Figure 1: Azides synthesized for use in azide alkyl cycloaddition.

8.1.1 Synthesis of azides 40-45

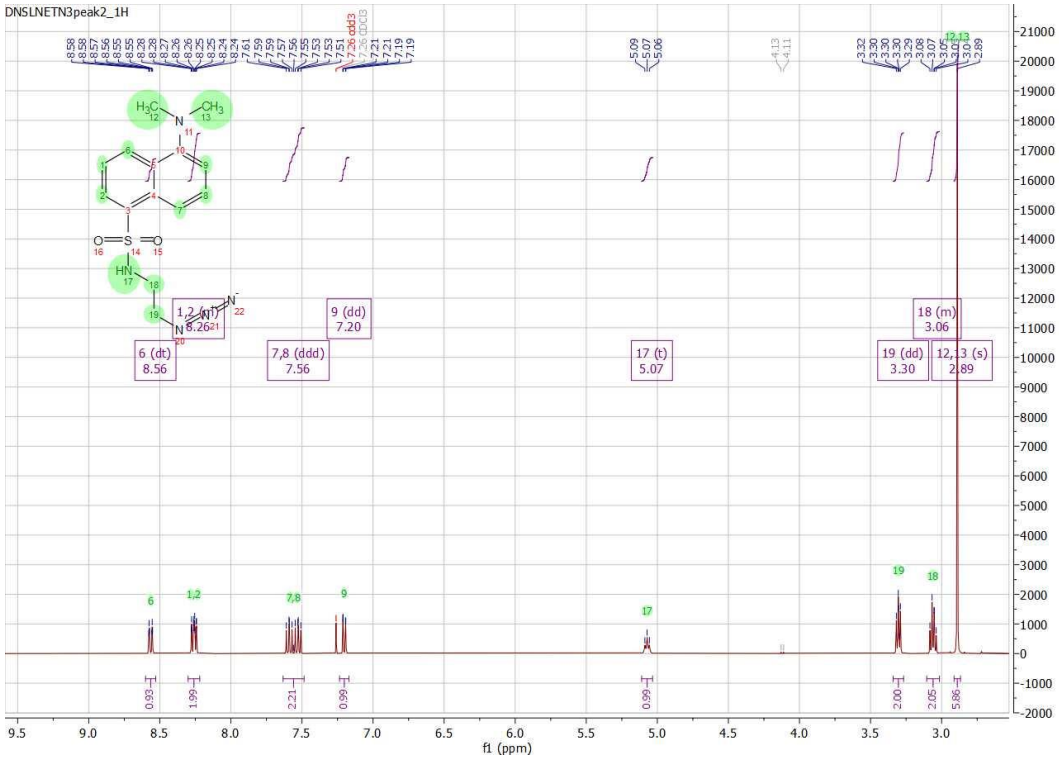
8.1.1.1 *Synthesis of N-(2-azidoethyl) naphthalene-1-sulfonamide (40)*

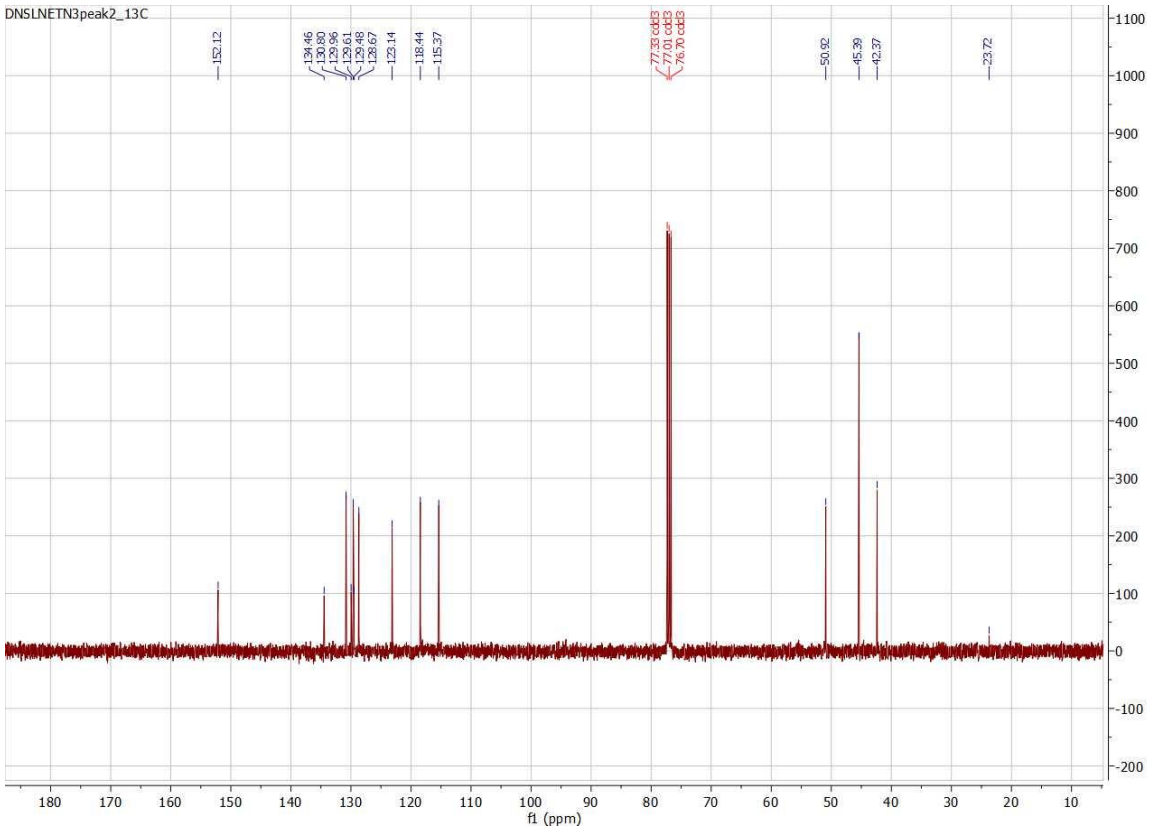
*8.1.1.1.1 NMR analysis of Compound **47**, intermediate in the synthesis of **40***

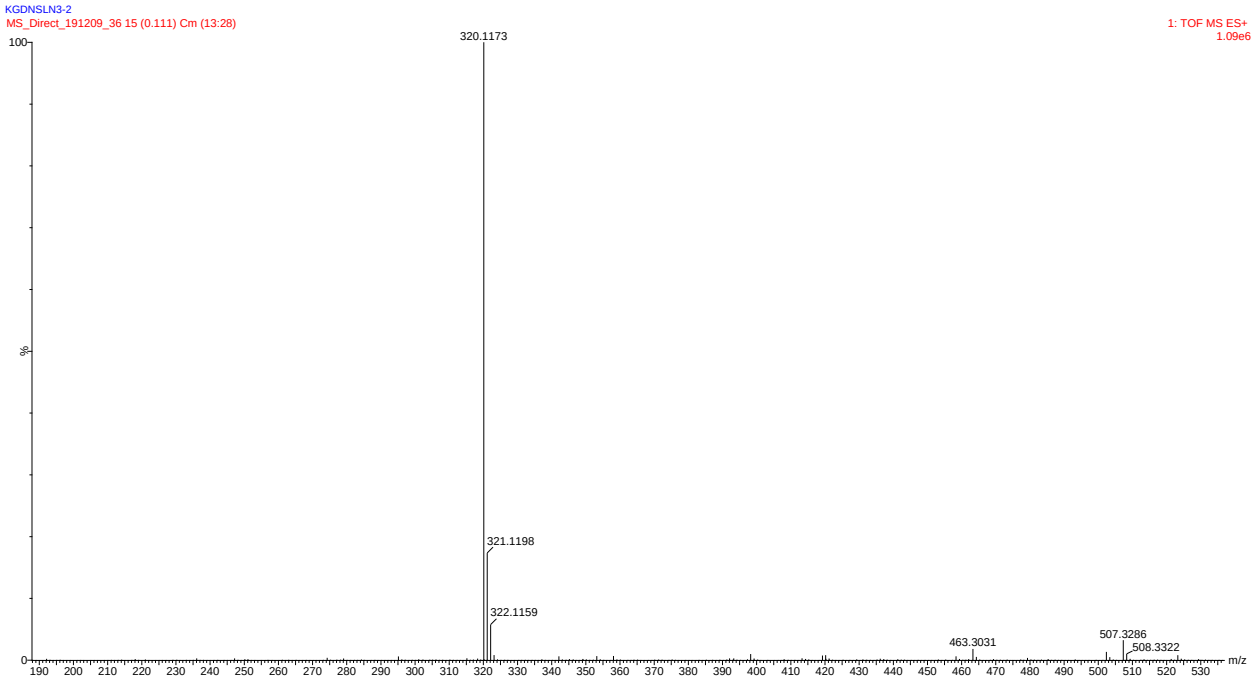


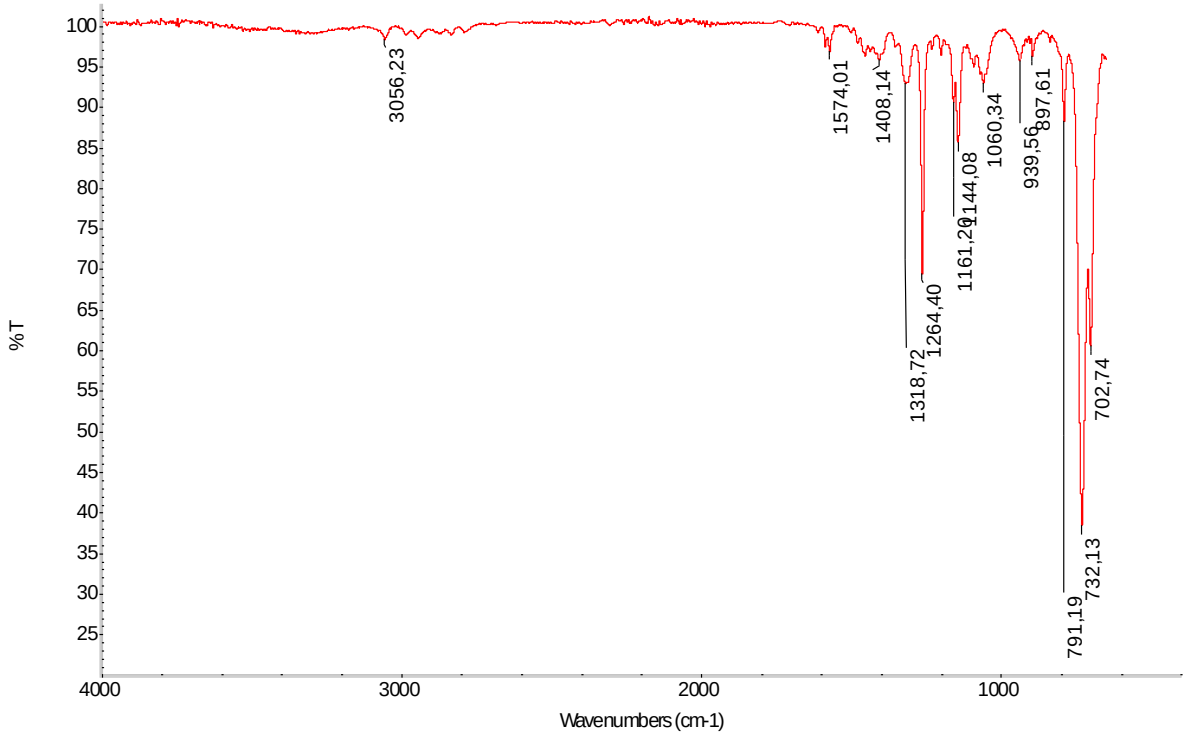


8.1.1.1.2 *Analysis of azide compound **40***



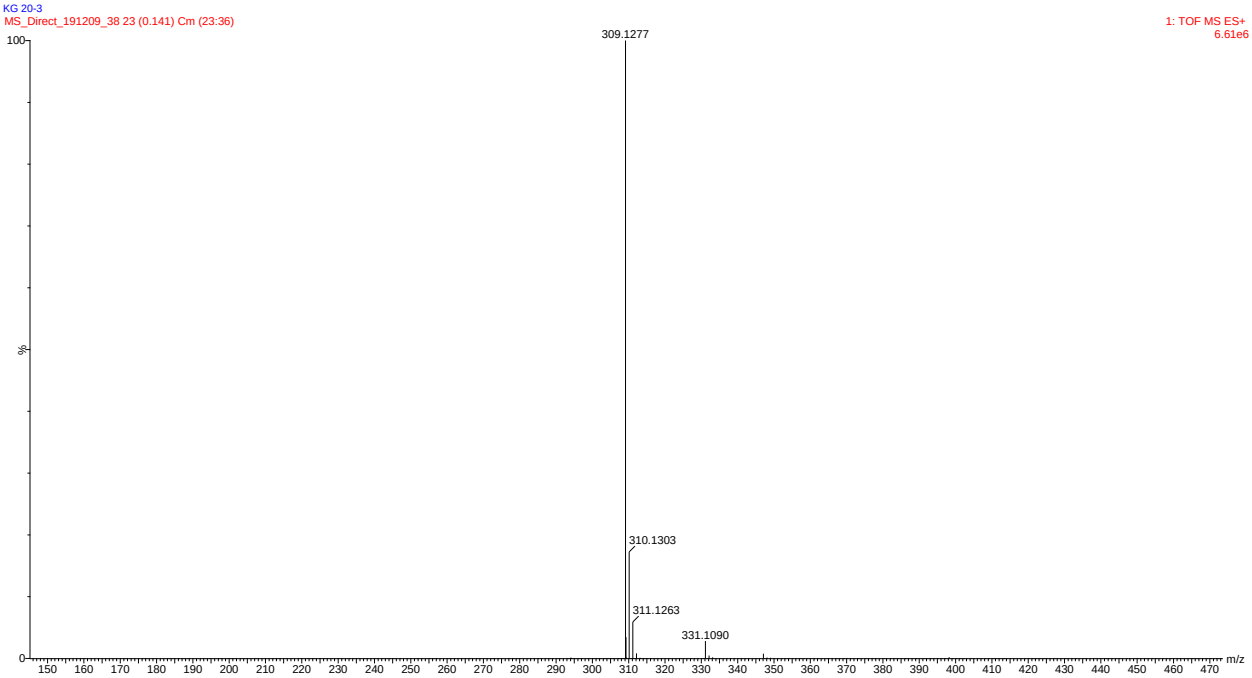


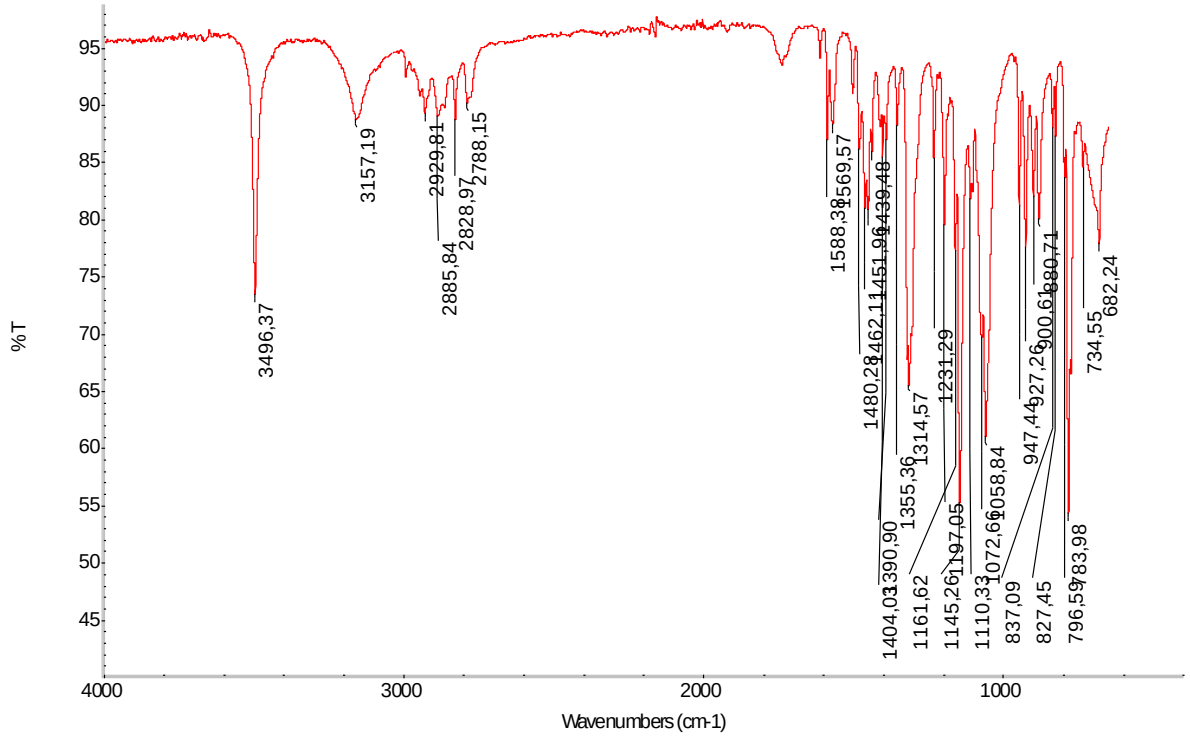




8.1.1.2 *Synthesis of N-(3-azidopropyl)-5-(dimethylamino)naphthalene-1-sulfonamide (41)*

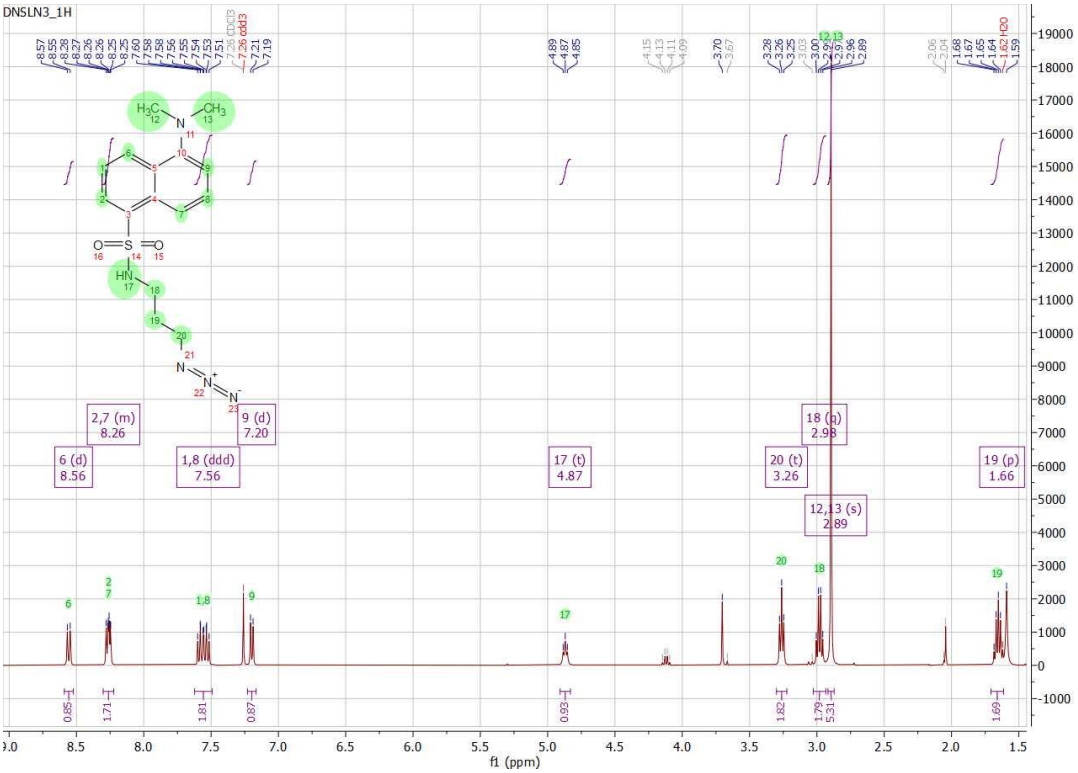
*8.1.1.2.1 Mass spectrum of Compound **49**, intermediate in the synthesis of **41***

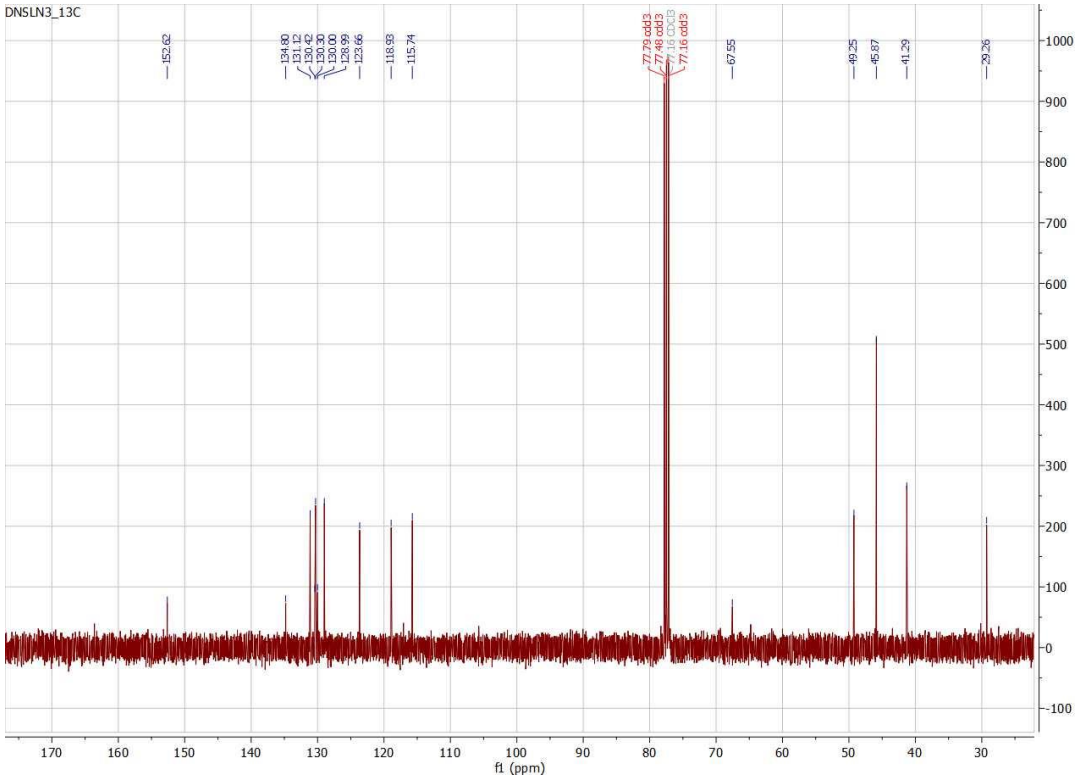


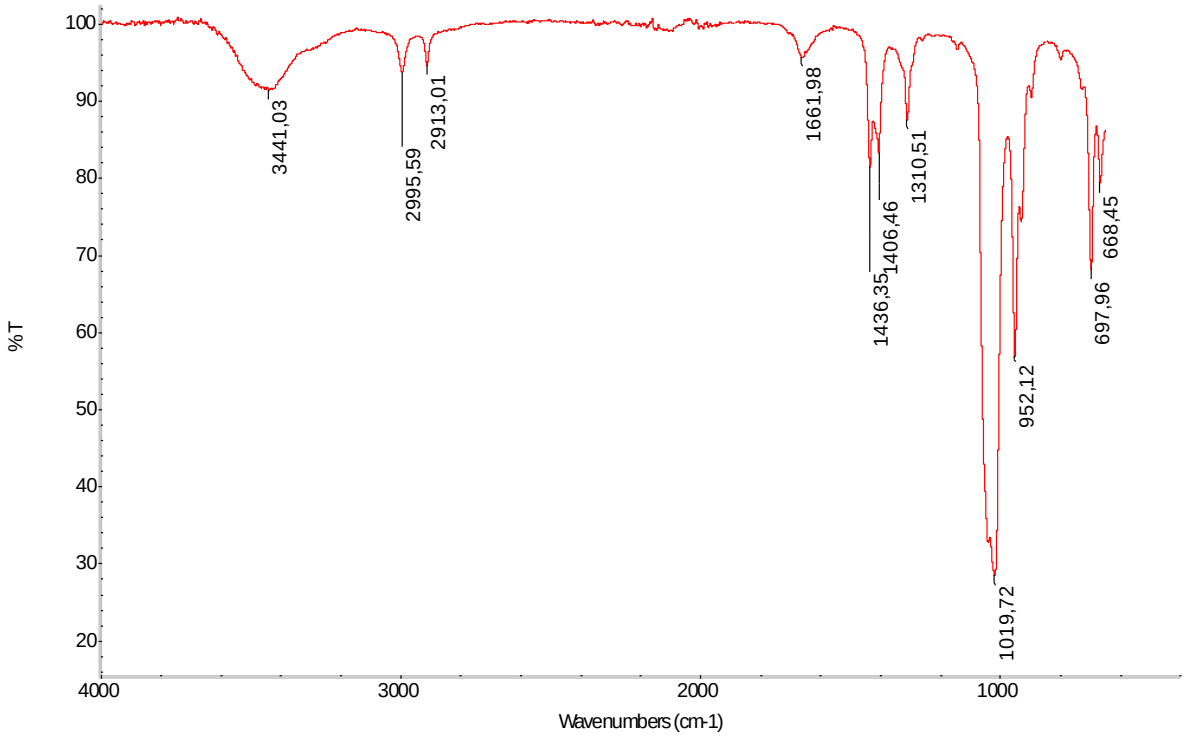


*8.1.1.2.2 Mass spectrum of intermediate Compound **50***

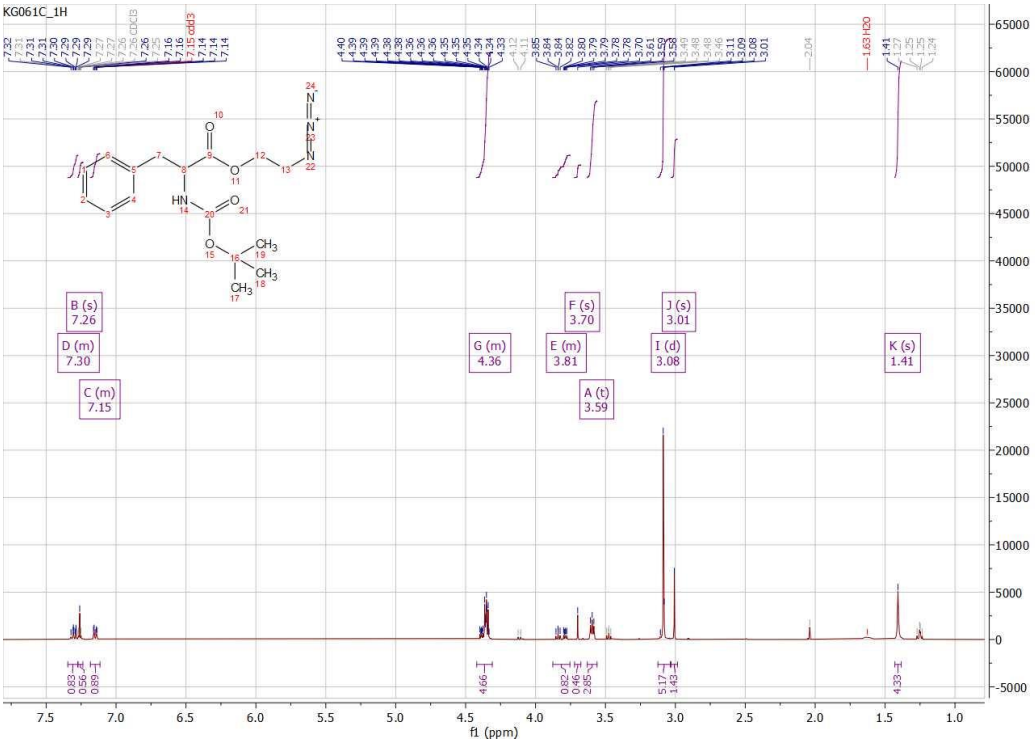
8.1.1.2.3 Analysis of compound 41:

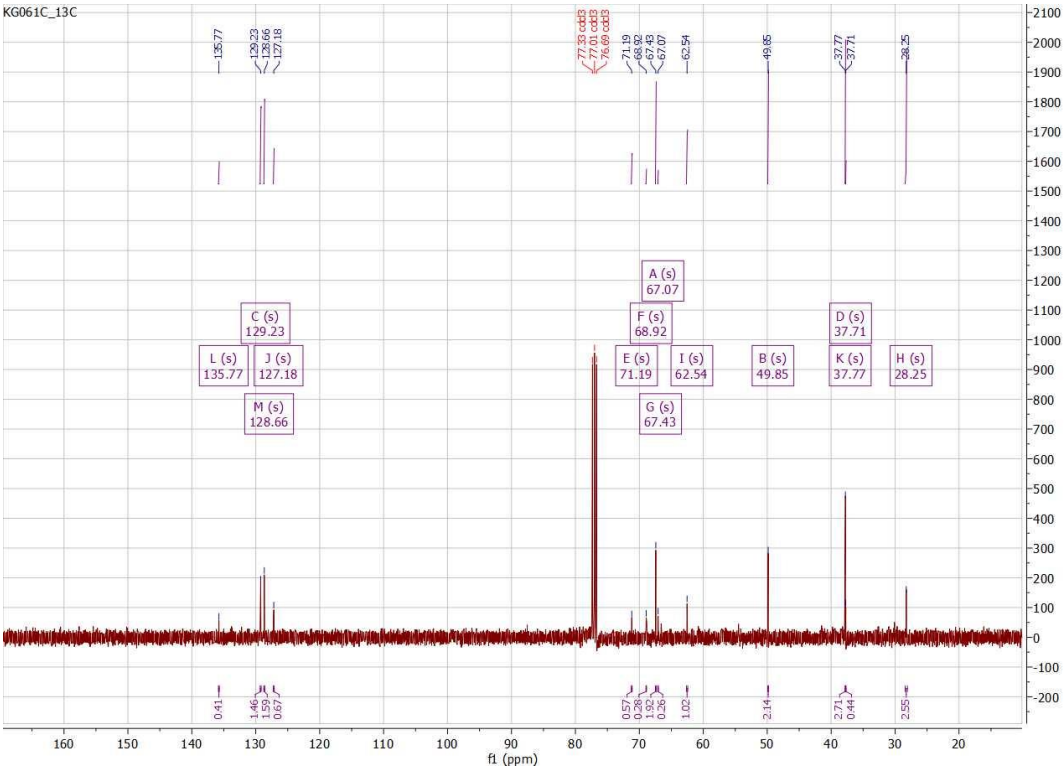


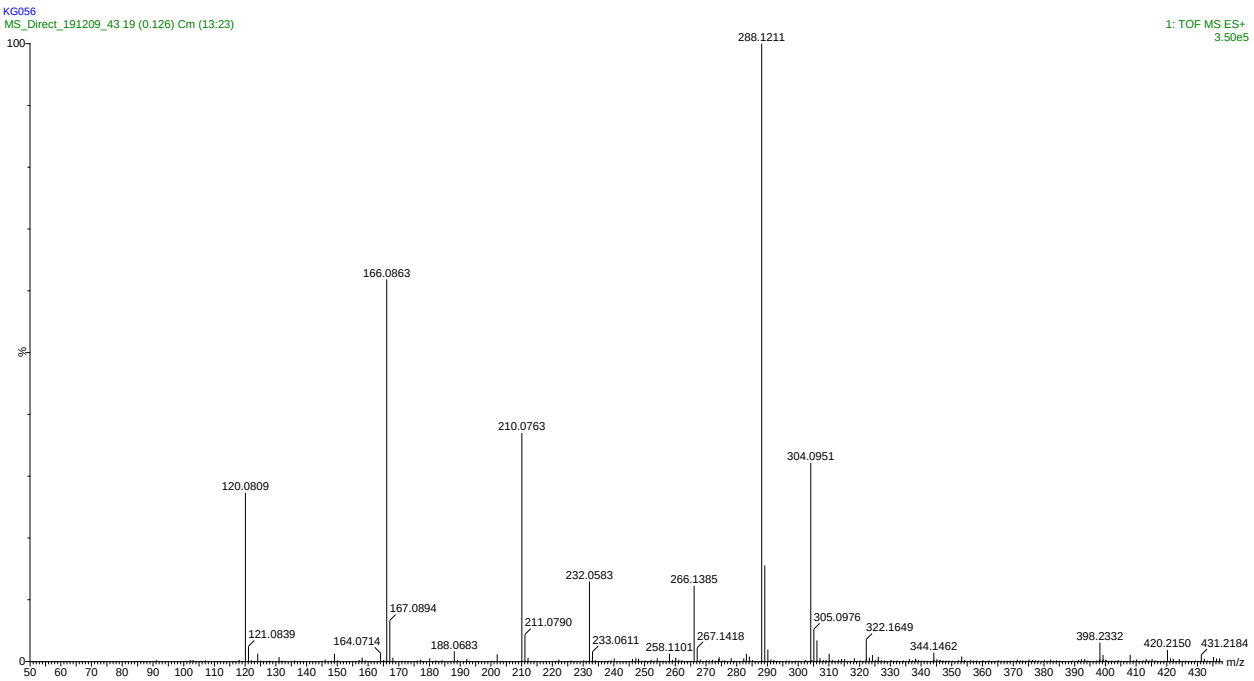


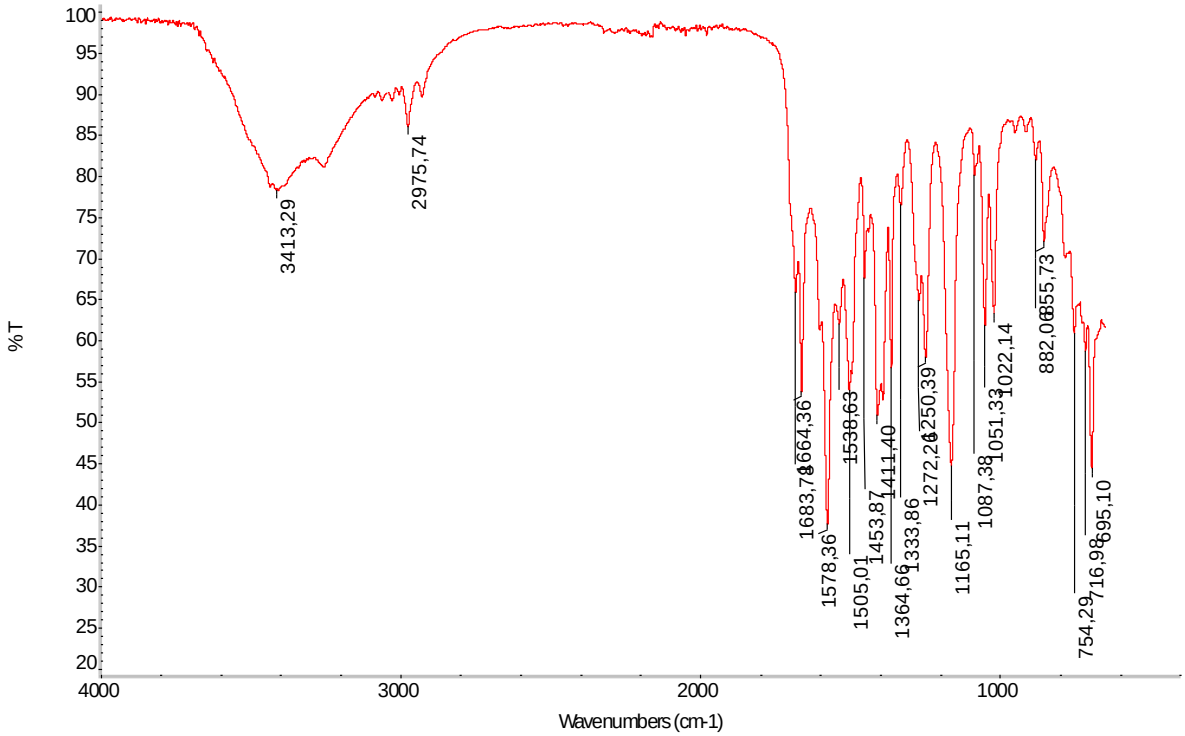


8.1.1.3 *Synthesis of (S)-2-azidoethyl 2-((tert-Butyldioxycarbonyl)amino)-3-phenylpropanoate (42)*





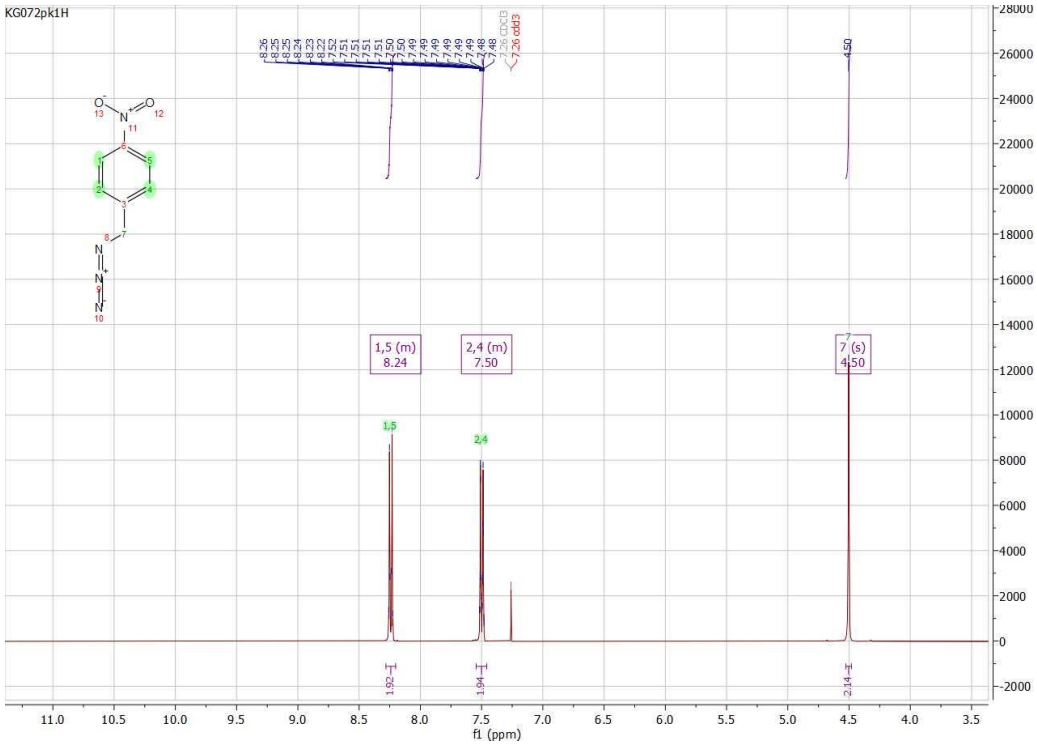




8.1.1.4 *Compound 43 and Compound 44*

These compounds were synthesized by a member of the Stellenbosch University Group of Organic and Medicinal Chemistry group MG Botes and analysis aligns with expected literature values.

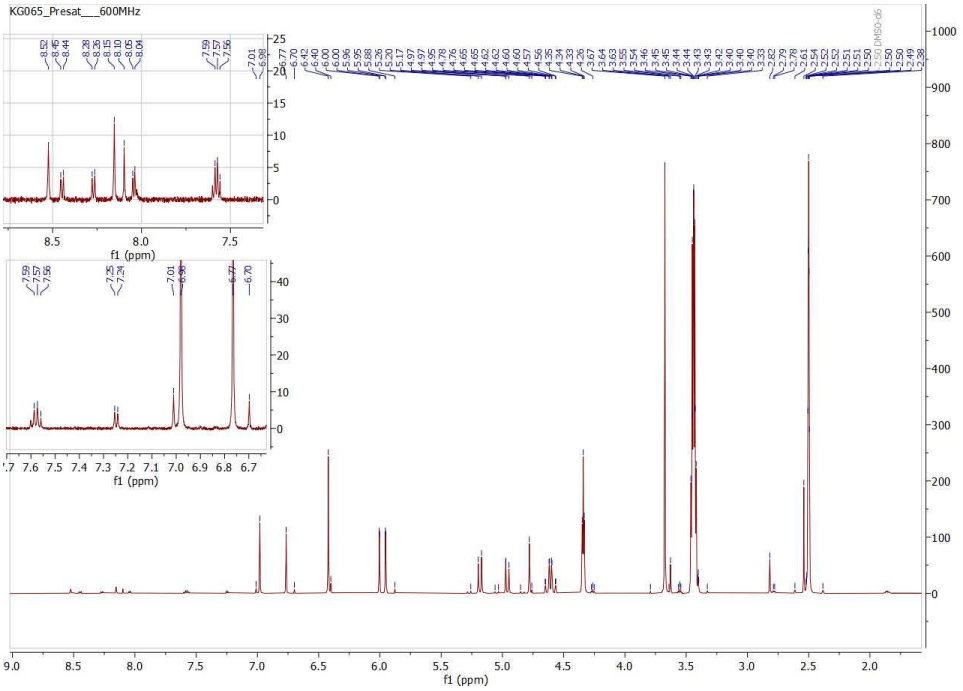
8.1.1.5 *Synthesis of 1-(azidomethyl)-4-nitrobenzene (45)*

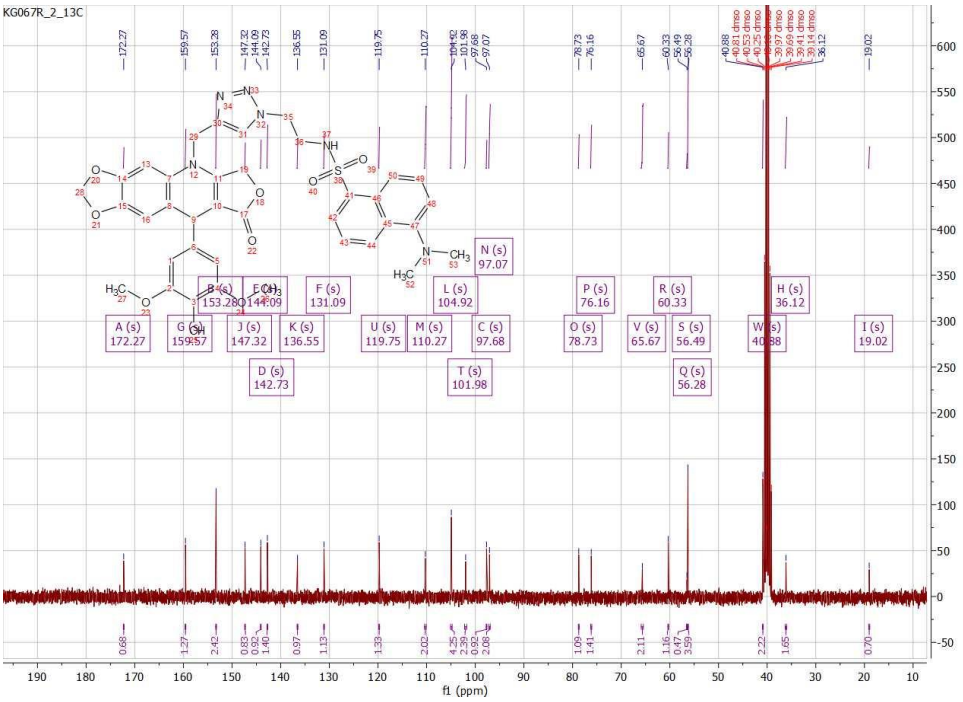


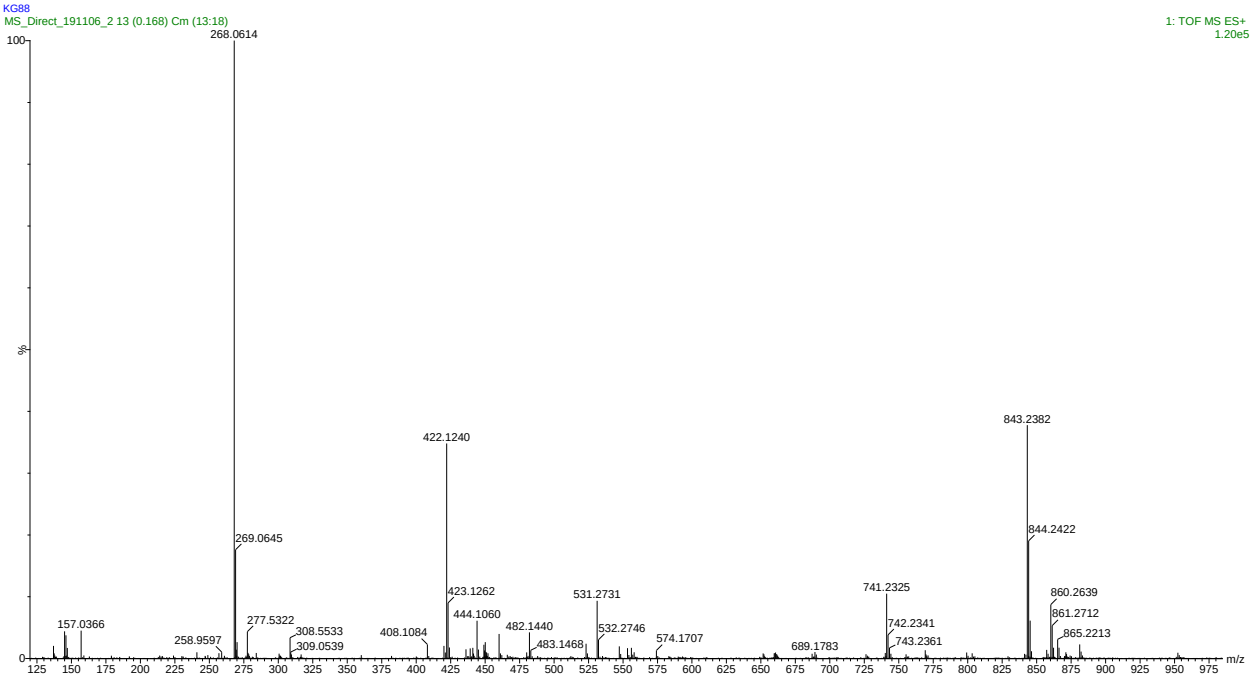
8.1.2 Synthesis of products 51a-56b

8.1.2.1 *Synthesis of 52 4N-aza-posophyllotoxin with fluorescent marker 40*

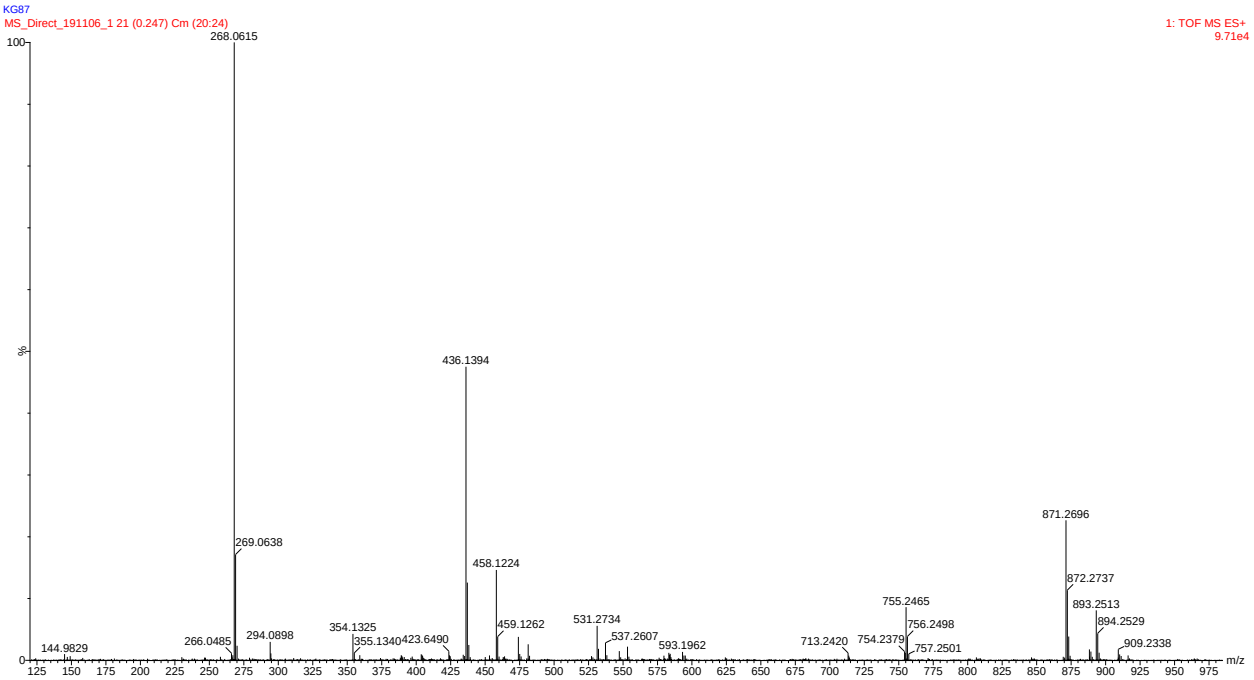
*8.1.2.1.1 Compound **52a***







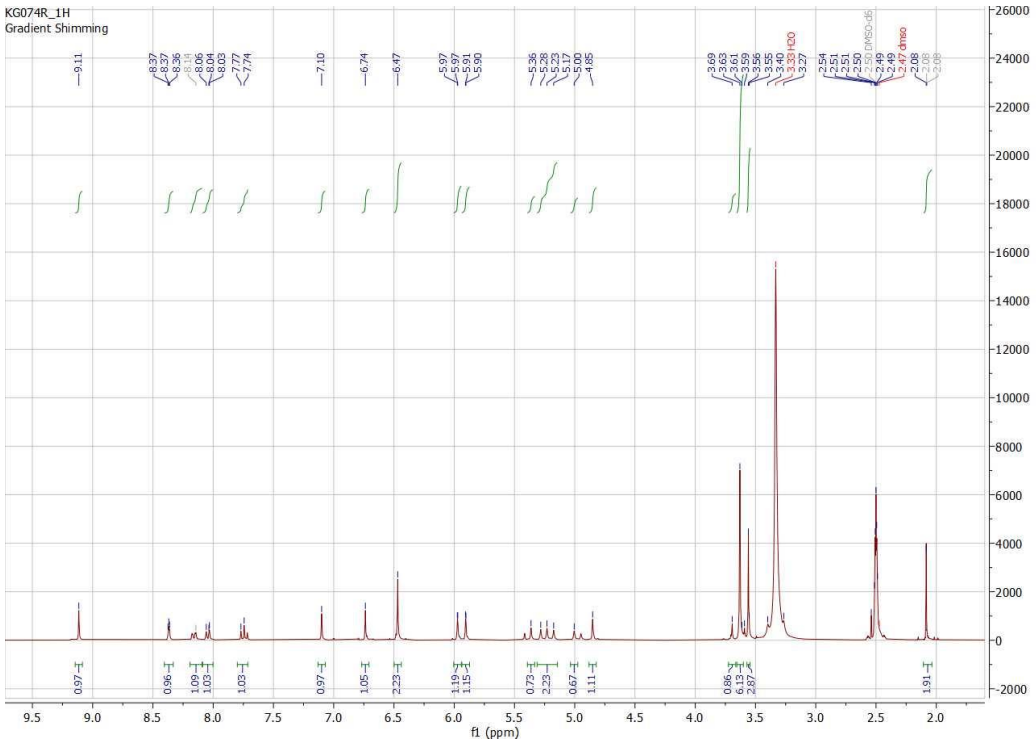
8.1.2.1.2 *Compound **52b***

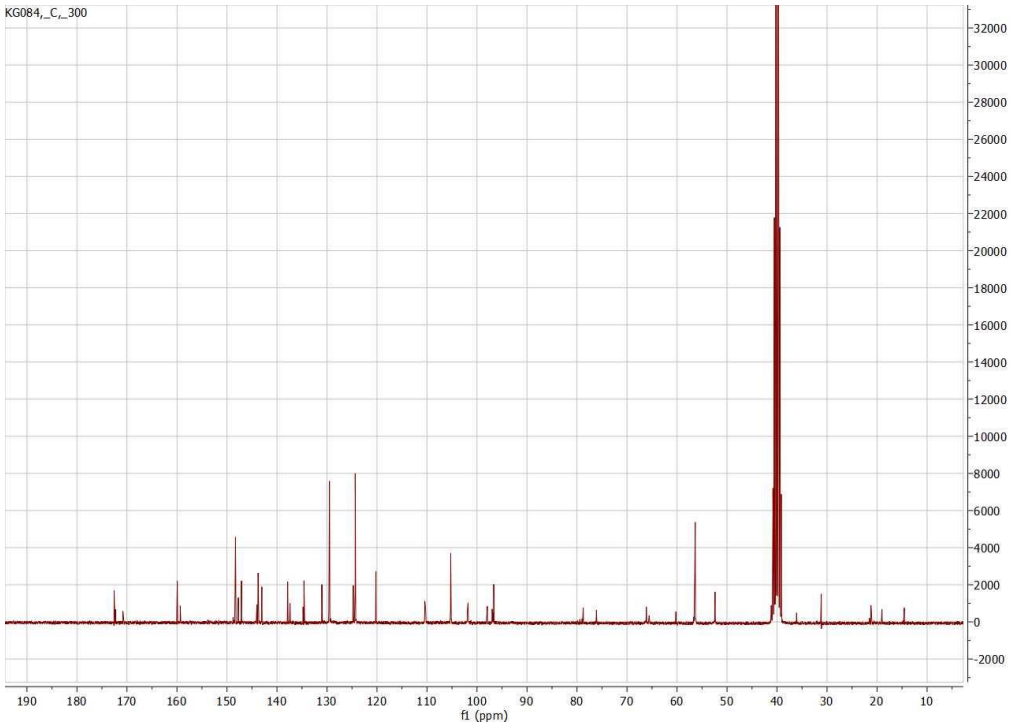


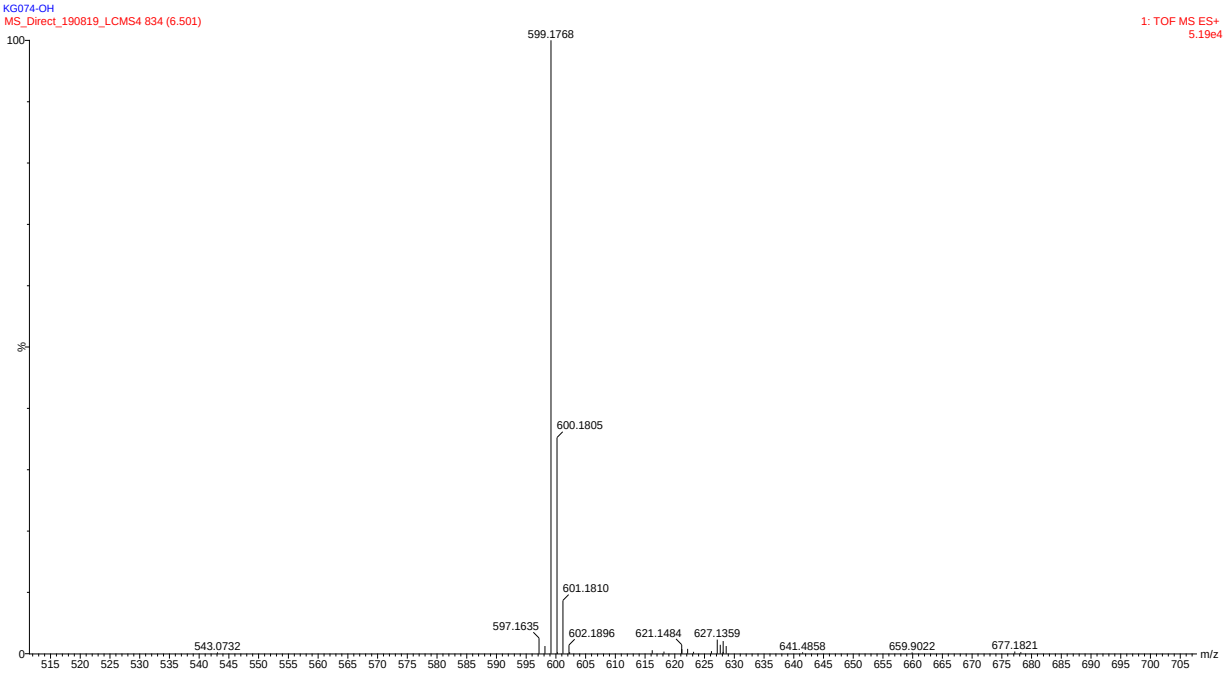
Compound **52b** was not observed with NMR spectroscopy and the starting material was recovered.

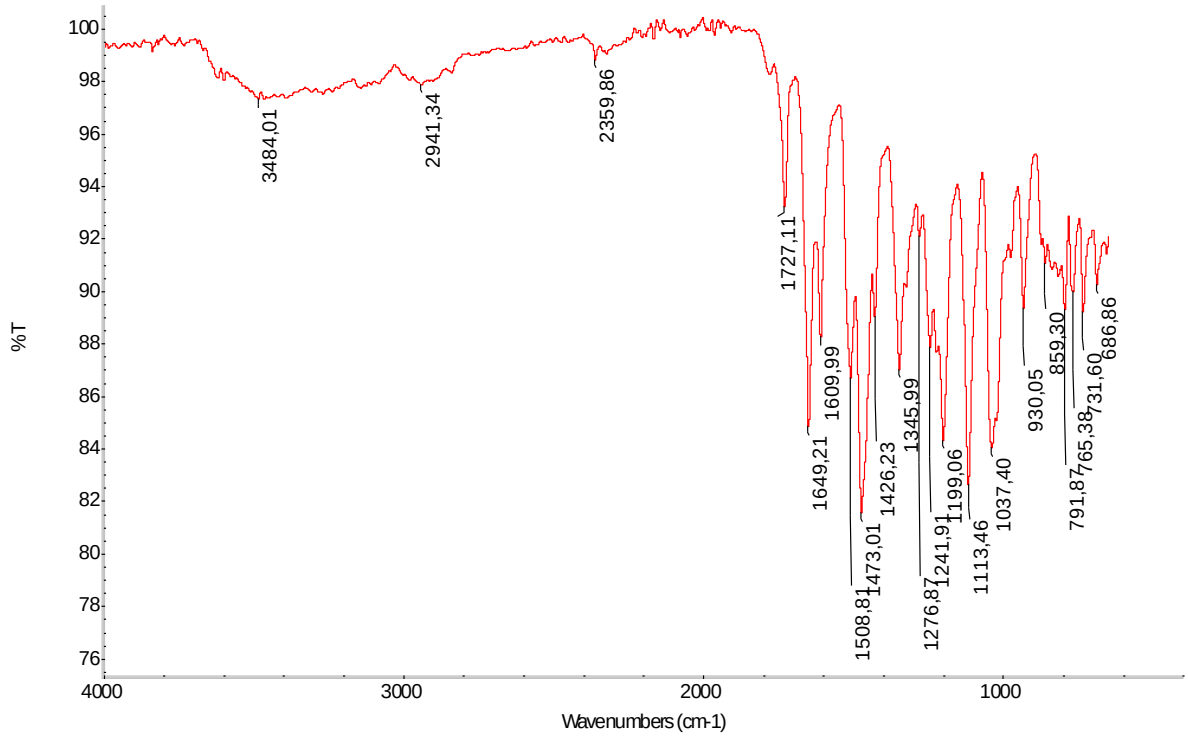
8.1.2.2 ***Synthesis of 54 4N-aza-podophyllotoxin with meta-benzylic acid***

*8.1.2.2.1 Compound **54a***







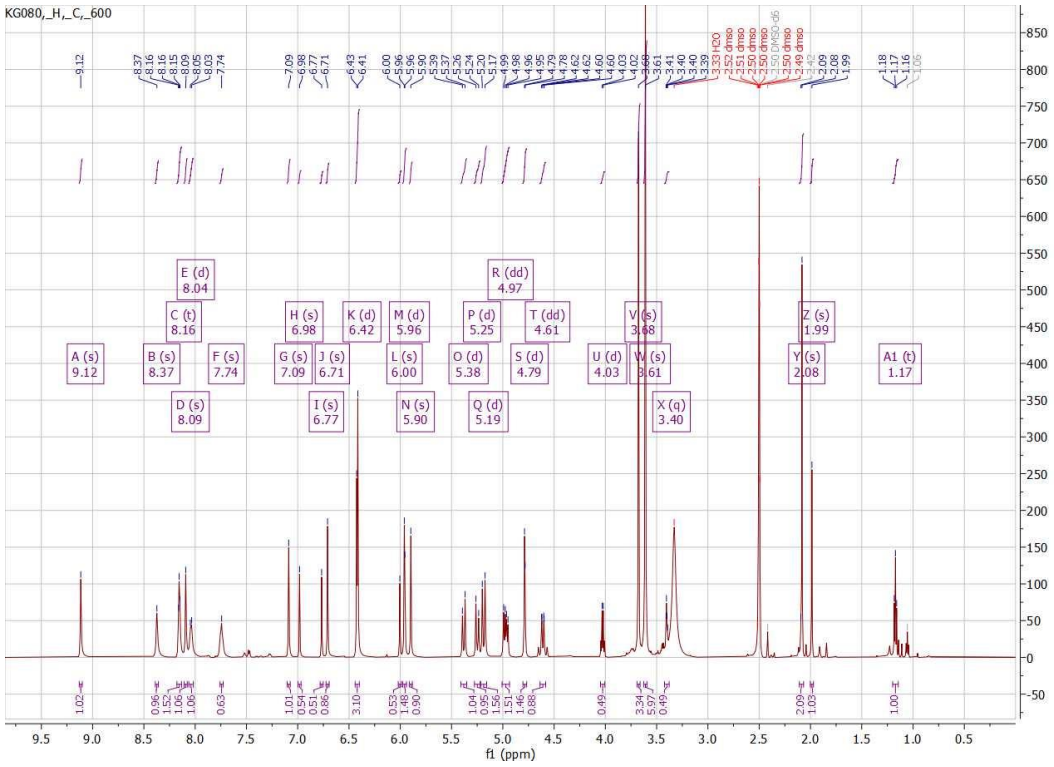


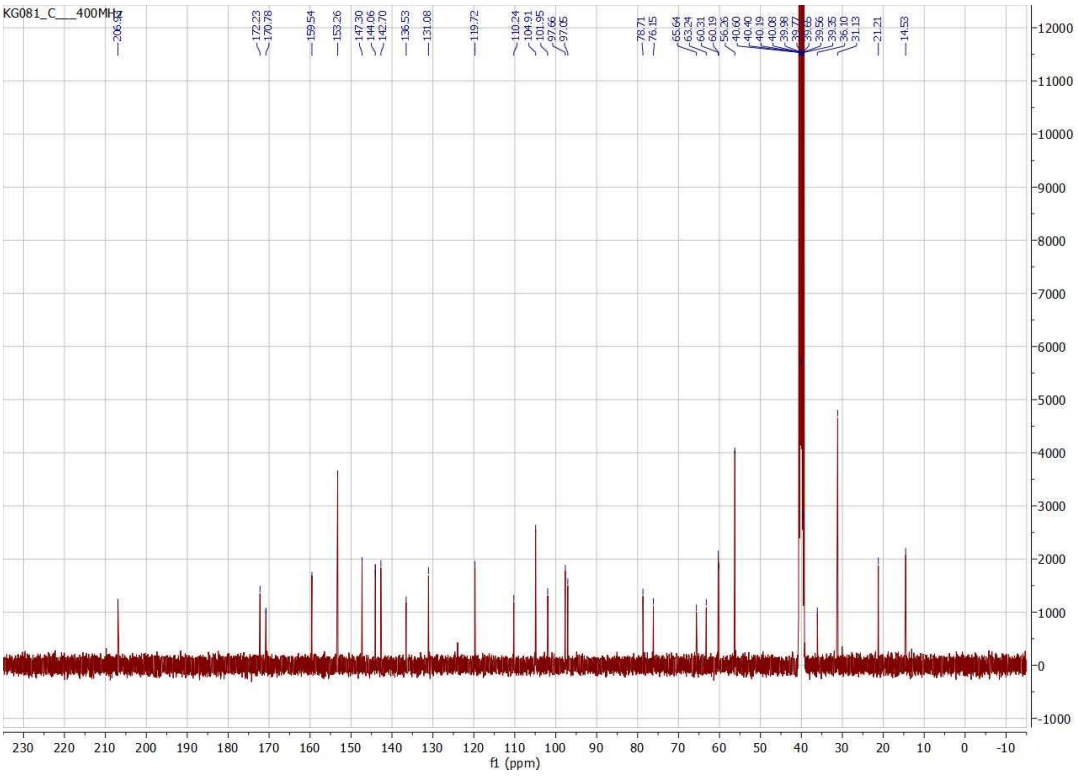
*8.1.2.2.2 Compound **54b***

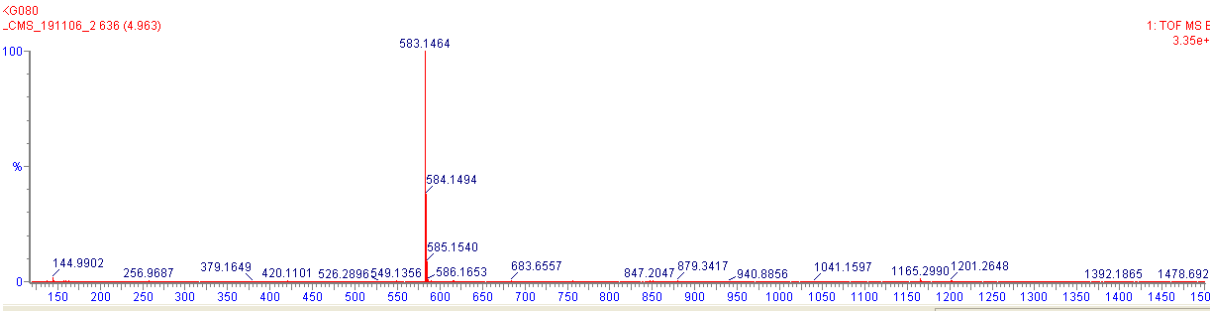
The compound was not synthesized in sufficient quantities for further analysis.

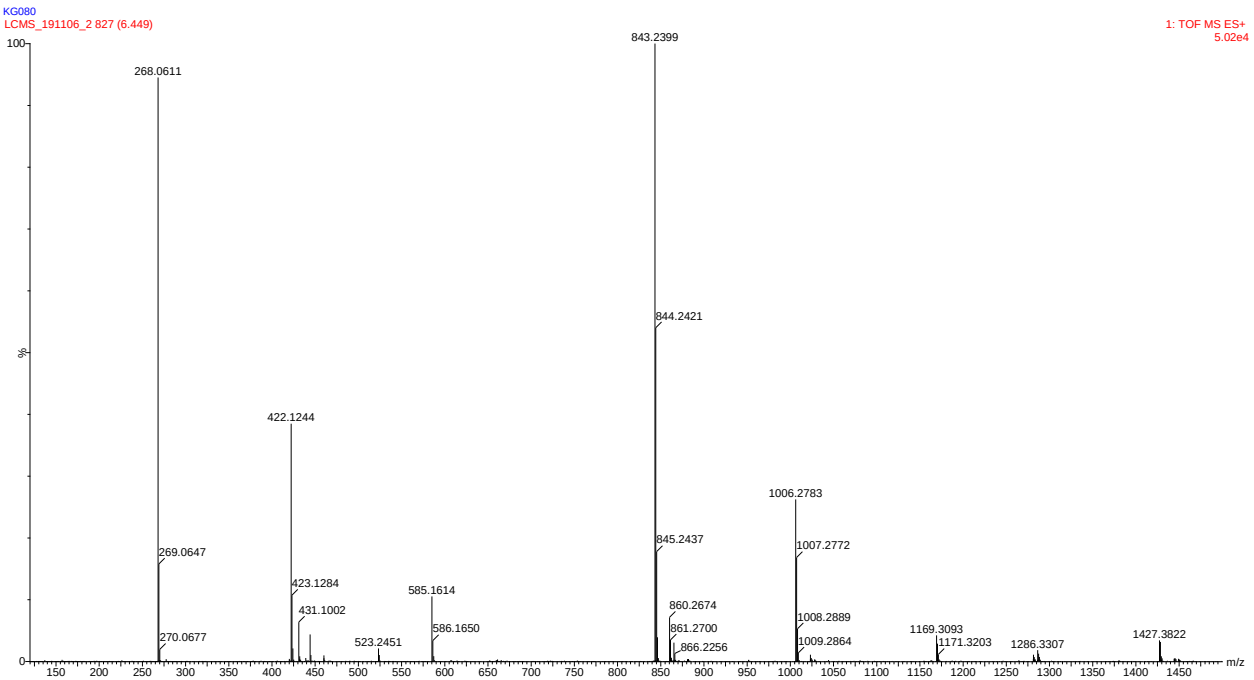
8.1.2.3 ***Synthesis of 55 4N-aza-podophyllotoxin with para-benzylic acid***

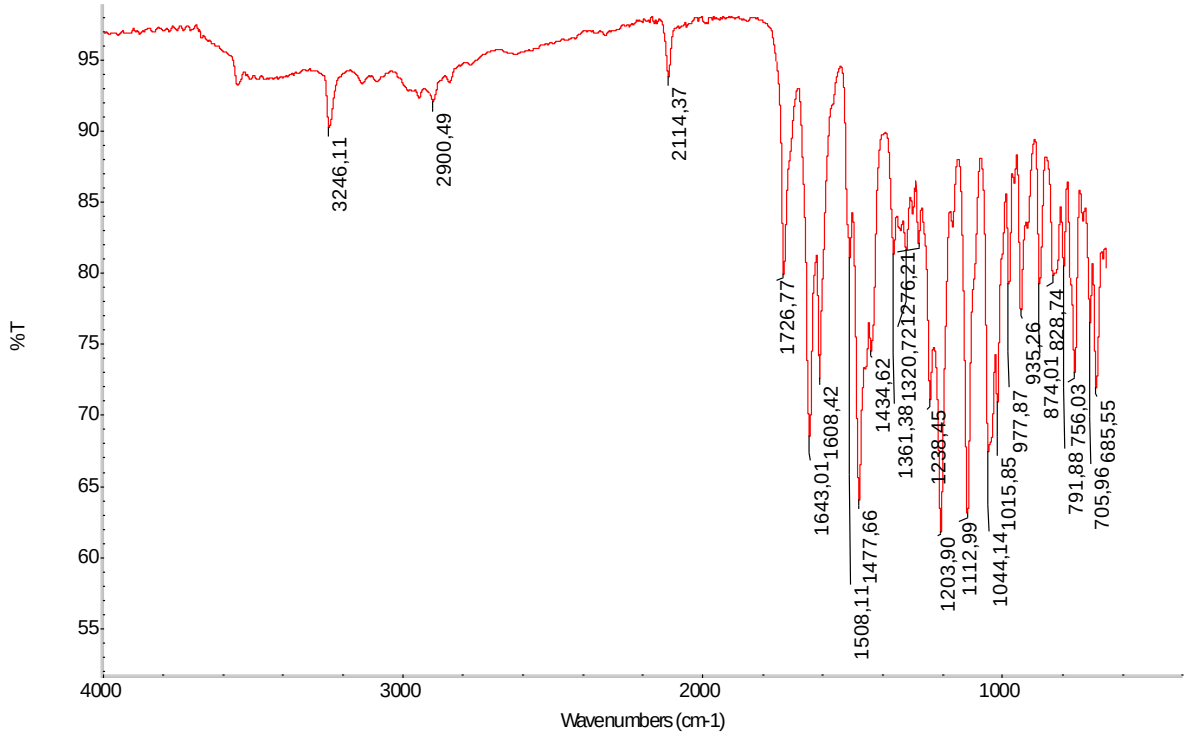
*8.1.2.3.1 Compound **55a***





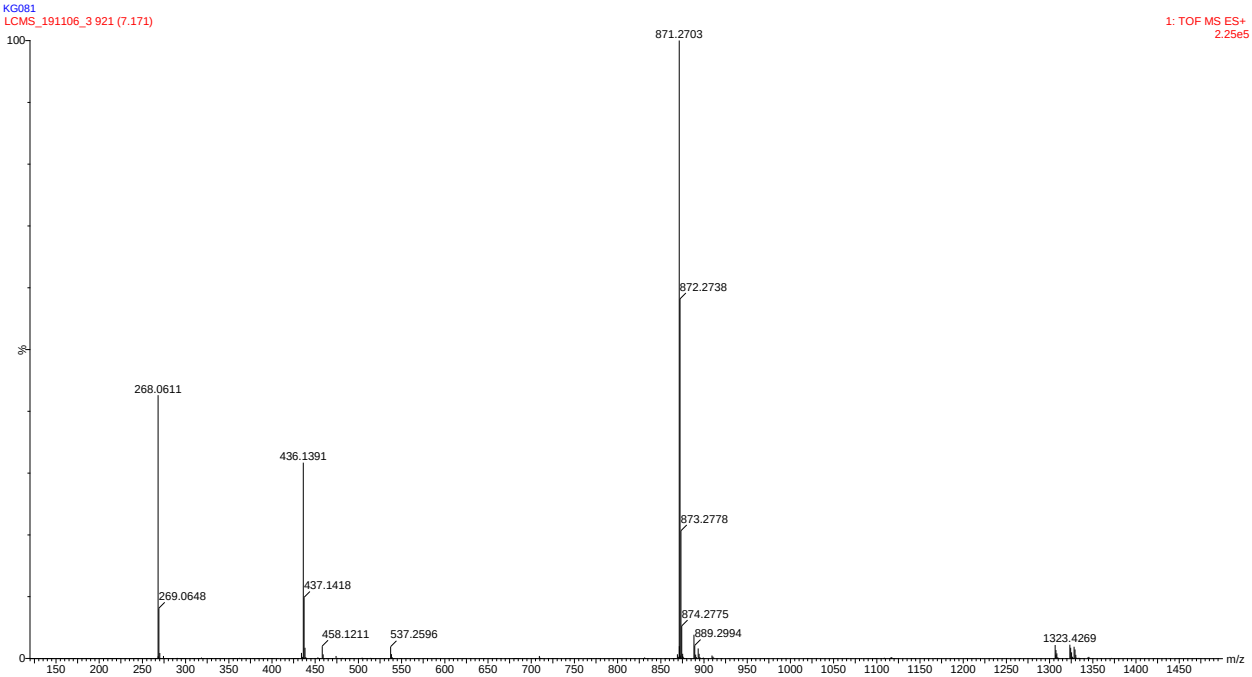


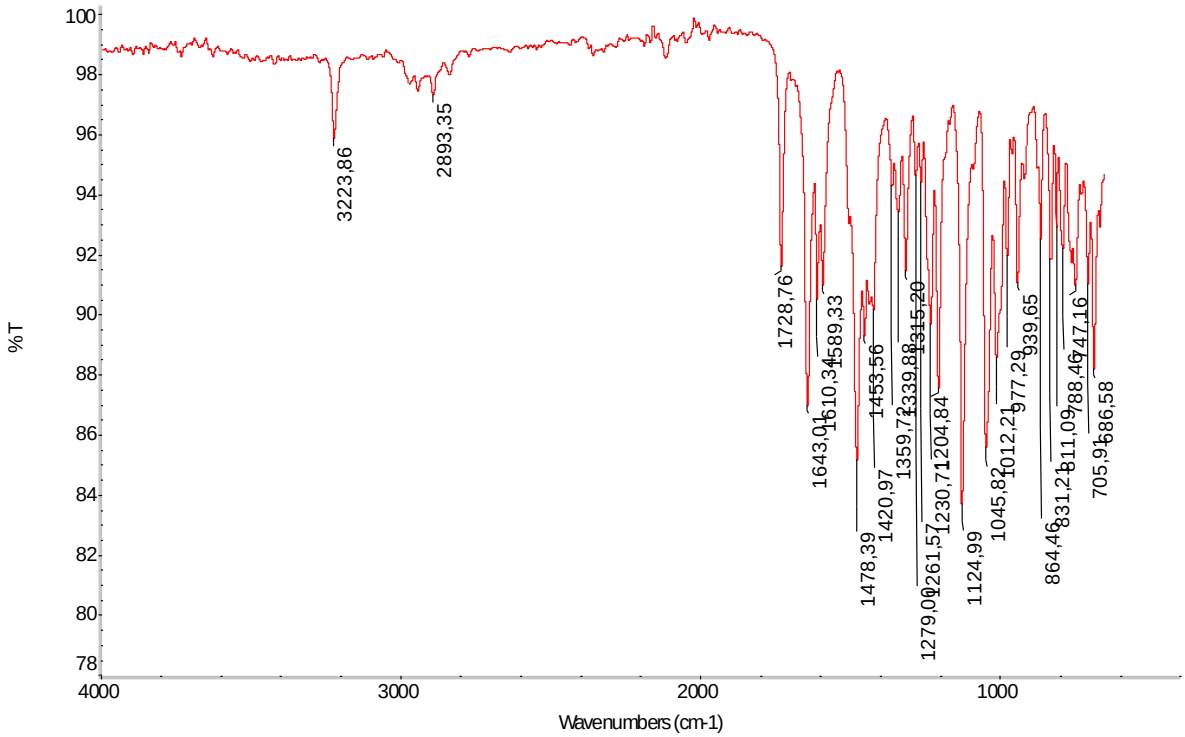




*8.1.2.3.2 Compound **55b***

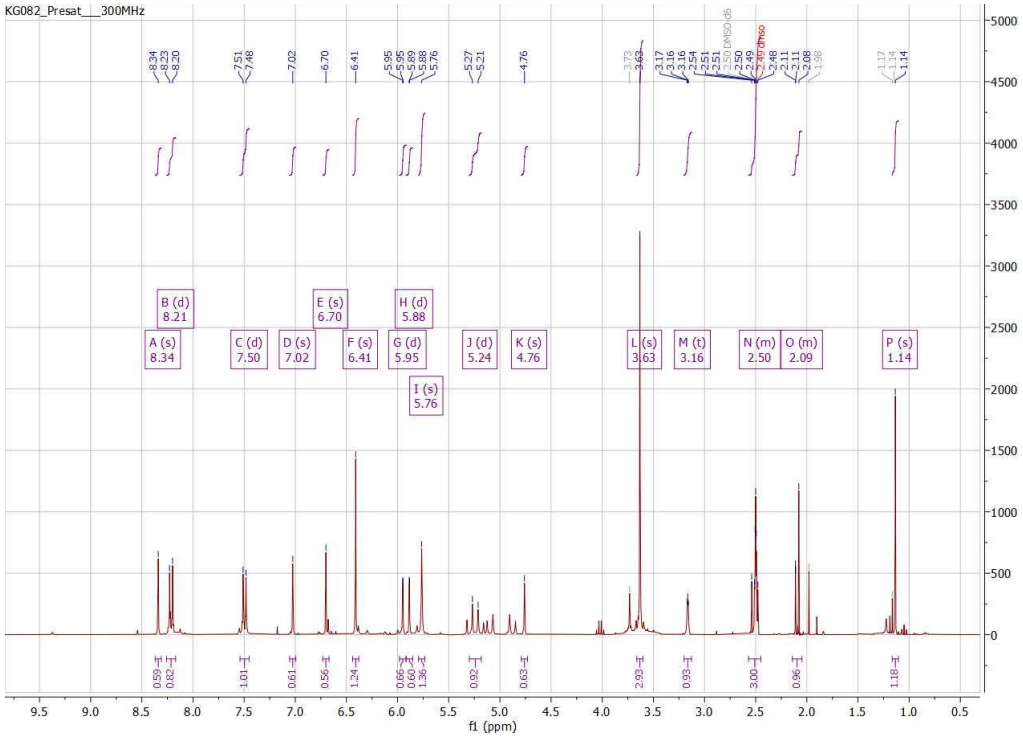
A polymeric mixture was observed

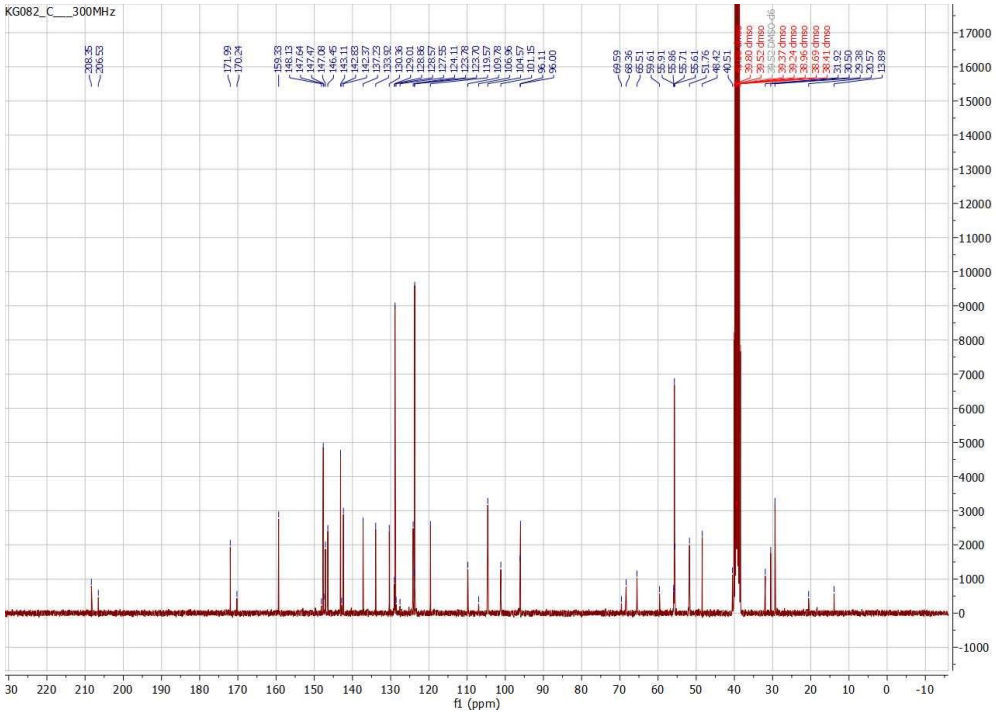


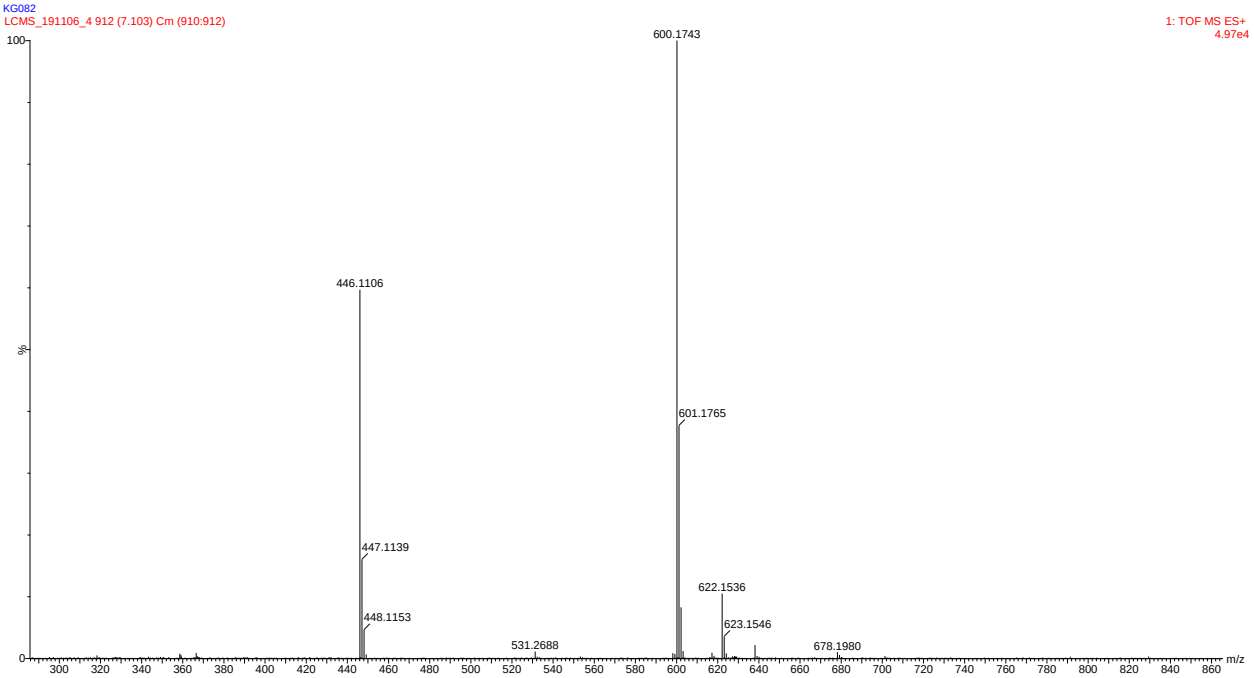


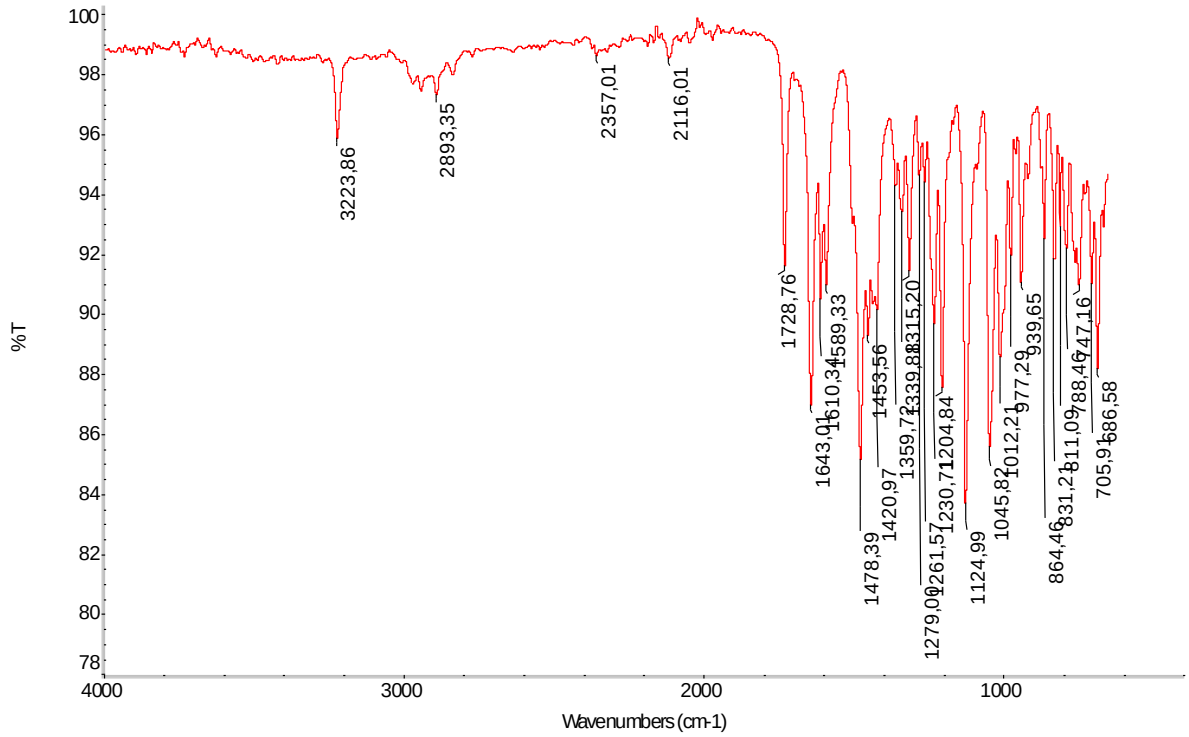
8.1.2.4 *Synthesis of 56 4N-aza-podophyllotoxin with 4-nitrobenzene*

*8.1.2.4.1 Compound **56a***









*8.1.2.4.2 Compound **56b***